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"Effects on the Human Respiratory System of Exposure to Selected Cotton Dusts."

Cooperators:

USDA, ARS, Cotton Quality Research Station, Clemson, SC
Henry H. Perkins, Jr., ADODR

University of Alabama at Birmingham
Principal Investigator: Dr. Robert R. Jacobs, PhD,
School of Public Health, University of Alabama at Birmingham

REPORT OF ACTIVITIES FOR
USDA COOPERATIVE AGREEMENT #58-43YK-0-0025.

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Executive Summary

In March of 1990 a five year cooperative agreement was developed between the United States Department of Agriculture and the University of Alabama at Birmingham. The overall objective of this agreement was to evaluate the acute response of naive subjects to aerosols of cotton dust and to determine if pre-existing indicators of atopy and non-specific bronchial reactivity can be used to identify those who are at increased risk to respond to cotton dust. A second objective of the study was an evaluation of the effectiveness of washing cotton on modern batch-kier systems for reducing the acute respiratory response to cotton dust. Additional objectives included: evaluation of the effect of dust generated from a synthetic textile fiber (rayon) on the ventilatory capacity and methacholine responsiveness of naive volunteers; evaluation the effect of cigarette smoking status on acute ventilatory responses to washed and unwashed cotton dust and rayon dust using basic spirometry and methacholine testing; evaluation of the carryover effect of an acute exposure to aerosols of cotton dust or rayon with a follow-up day exposure to rayon; and comparison of the acute ventilatory response to a washed:unwashed blended preparation of cotton dust with a level of endotoxin equivalent to an unwashed California dust. Other peripheral studies included: four student projects at the University of Alabama at Birmingham and a microbiologic and endotoxin evaluation of bulk fiber samples and airborne samples of card generated dust from cottons and rayon.

For all studies, healthy volunteers without respiratory symptoms and normal ventilatory capacity by spirometry were studied with a standard clinical test of lung function before and after a 5-hour exposure to a controlled level of cotton dust. The cotton dust level chosen for most of the studies was 1 mg m^{-3}. This level of dust has been shown to produce mild transient decreases in the ventilatory capacity of about 25% of healthy volunteers. In some of the studies airway reactivity was also evaluated before and after the exposure to cotton dust using a standard technique consisting of inhalations of graded doses of methacholine. For each study year the atopic status of the participants was determined subjectively by questionnaire and objectively confirmed either by skin prick testing or by total IgE Rast. Based on the data from the objective test the subjects were categorized as atopic or non-atopic and exposure groupings developed which contained approximately equal numbers of atopic and non-atopic subjects. In the first three years of the study nasal lavage was done on subjects before and after their exposure to cotton dust.

In 1990, atopic subjects had a significantly greater mean fall in FEV_1 after exposure to cotton dust than non-atopics (8.3% vs 4.9% $p<0.001$). There was also a significant increase in airway reactivity in both atopics and non-atopics (68% vs 20% reaching a PD_{20}, $p<0.0005$) after cotton dust exposure. These studies demonstrated that both atopics and non-atopics respond to cotton dust with significant drops in ventilatory measures (FEV_1) and significant increases in non-specific airway reactivity. Atopics had significantly larger decreases in FEV_1 than non-atopics and, while the proportionate increase in non-specific airway reactivity was similar for both atopics and non-atopics, the baseline reactivity of atopics was significantly higher than non-atopics suggesting that the risk for both acute and chronic impairment from an exposure to cotton dust may be greater for atopics.

Results from the studies in 1991 demonstrated that both atopic and non-atopic subjects, exposed to aerosols of cotton dust with different levels of endotoxins, had significant increases in non-specific airway reactivity and significant changes in acute ventilatory parameters. Atopics tended to have greater drops in FEV_1 and increases in methacholine responsiveness than non-atopics, however, unlike 1990, these differences were not significantly different. Although the changes were small, both atopic and non-atopic subjects had increased airway reactivity and acute pre to post-exposure changes in FEV_1 to aerosols of dust from rayon. These data indicate that rayon is not an inert dust. Washing cotton did remove the acute ventilatory response (FEV_1) relative to exposure to rayon, however washing did not remove the methacholine responsiveness relative to rayon when subjects exposed to washed cotton at 1 mg/m^3. When the exposure level of washed cotton was reduced to 0.5 mg/m^3, both the acute ventilatory response (FEV_1) and methacholine responsiveness were no different that the response to rayon. In general, there was good agreement between the level of endotoxin and magnitude of change in FEV_1, however, the endotoxin did not correlate with the magnitude of change in non-specific airway reactivity.

Studies in 1992 demonstrated that smoking naive atopic and non-atopic subjects responded to aerosols of cotton dust with increases in non-specific bronchial reactivity and over-exposure changes in FEV_1. Again, atopics tended to be more responsive than non-atopics, but the differences in ventilatory response and methacholine response between the two groups were not significant. For smokers, exposure to washed cotton at 1 mg/m^3 did not cause a change in FEV_1 or methacholine responsiveness that was significantly different from exposure to rayon. These data were different than that observed in 1991 for non-smokers and suggest that smokers were less responsive to aerosols of cotton dust and rayon that non-smokers.

Studies in 1993 demonstrated that the increased non-specific airway responsiveness caused by an acute exposure to cotton dust does not cause an increase in responsiveness, as measured by an acute change in FEV_1, to rayon. While atopics again tended to be more responsive than non-atopics the differences were not significant.

In summary, these studies demonstrated that atopics tend to be more responsive to aerosol of cotton dust than non-atopics. However, the differences between atopics and non-atopics are small and atopic status has limited use as a risk factor for predicting responsiveness to cotton dust. Washing cotton removes the ventilatory activity of cotton dust, however, for non-smoking subjects it does not completely remove the airway reactivity as measured by methacholine responsiveness. The level of endotoxin in cotton dust correlates more strongly with the potential to cause changes in acute ventilatory measures than with changes in non-specific airway reactivity as measured by methacholine responsiveness. The acute increase in non-specific airway reactivity caused by an exposure to cotton dust does not enhance the response to a follow-up exposure rayon.

Background

Both acute and chronic effects on the respiratory system have been attributed to the inhalation of cotton dust in the work place (1-3). However, these effects are poorly understood. Some workers exposed to dust from cotton, flax, or hemp experience a sensation of chest tightness or shortness of breath on the first day of the workweek. These symptoms generally remit spontaneously after work and do not return during the rest of the week despite continued exposure. Some but by no means all of these symptomatic workers also show decrements in lung function during the workshift. The most well documented is a mild decrease in the forced expiratory volume in one second (FEV_1), the amount of gas expelled during the first second of a maximal effort exhalation from full inhalation (4,5). The FEV_1 generally returns to the pre-exposure level by the following day and does not show a progressive decline during the workweek. The acute decrease in FEV_1 is felt to reflect a narrowing of the airways in response to one or more active agents in the dust. The exact nature and mode of action of these agents have not been identified (1). However, it seems clear that more than just a direct "irritant" effect of the inhaled particles is involved since water washing of cotton prior to processing seems to prevent the decrement in FEV_1 during exposure (6,7). Unfortunately water washing of cotton does not appear to be feasible for most segments of the textile industry for both economic and technical reasons. Control measures have concentrated on lowering dust levels in the workplace and attempting to identify those at risk of suffering long-term adverse health effects from exposure by medical surveillance. However, evidence has been presented that some persons will manifest acute responses at dust levels lower than currently achievable (8). Thus, the interpretation of medical surveillance results is not straight forward and appropriate action to protect the health of workers is often in question.

Chronic effects of the inhalation of cotton dust on the respiratory system are also poorly understood (1). Some persons exposed for several years appear to develop a decrement in lung function which is not rapidly reversed by cessation of exposure (9,10). This may be due to structural changes in the airway walls consisting mainly of an increase in the size of mucous secreting glands, thickening of the muscular component, and chronic inflammatory cell infiltration together described as chronic bronchitis (11). Those who smoke cigarettes and also are exposed to cotton dust appear most like to develop chronic bronchitis but non-smoking textile workers are also at risk. Prospective studies of groups of textile workers have provided evidence that their ventilatory capacity decreases at a greater annual rate than non-textile workers (12, 13). It is not known whether those who show acute symptoms or decrements in function over a workshift are definitely at greater risk of developing chronic loss in function although some studies have provided evidence that this may be the case (51,56). If so, this makes precise characterization of the acute response important for accurately identifying those at risk as well as for investigating the mechanisms of action of specific agents that may be involved in the response.

A major obstacle in the path of advancement of understanding of the mechanisms and risk factors for effects of cotton dust inhalation is the lack of adequate characterization of individuals showing the acute response. Only 25% of the healthy volunteers screened for participation in model cardroom studies showed a significant drop in FEV_1 during six hours of exposure to 1

mg/m^3 of airborne cotton dust (14). While investigations of textile workers have not found evidence that the response to cotton dust is immunologically mediated (15), studies have reported that HLA-B27 (human leukocyte antigen) antigen frequency was increased in flax workers with byssinosis (16) and that workers categorized as atopic by positive skin test and personal history showed a greater pulmonary response than non-atopic workers in the early stages of cotton processing (17). These findings imply a variability in the susceptibility of the population and a question of interest is one of defining the sources of this variability in responsiveness and of characterizing markers that can identify susceptible individuals.

Two potential markers that may be related to the variability of the acute response to cotton dust are non-specific bronchial responsiveness and atopic status. Persons with the clinical picture of asthma can be shown to have an increased bronchial responsiveness even during periods of apparent remission (18). Inhaled histamine causes airway narrowing by stimulation of irritant receptors and perhaps by direct action on smooth muscle (19). Asthmatics manifest a given degree of narrowing at much lower concentrations of histamine than normals. This is not an immune system, i.e., allergic response itself, but an indicator of the increased responsiveness to many non-specific stimuli. In a study conducted for NIOSH by one of the co-investigators for this proposal (Boehlecke), volunteers were tested for bronchial reactivity and the following week were exposed to airborne cotton dust at 1 mg/m^3 for six hours with measurement of FEV_1 before and after exposure (20). Although a weak trend was present no significant association was found between the drop in FEV_1 during cotton dust exposure and the previously measured bronchial reactivity. This study was limited by the separation of several days between the cotton dust exposure and the measurement of bronchial reactivity. No post-cotton dust exposure measurement of bronchial reactivity was carried out. Others have found mixed results when attempting to correlate non-specific bronchial reactivity with responsiveness to inhalation of aerosols of water extracts of cotton plant parts (21,22). They found a correlation when non-specific bronchial reactivity was measured using methacholine, a compound related to the neurotransmitter acetylcholine, but did not find an association when using histamine. However, the inhalation of water soluble extracts of cotton plant parts may not be equivalent to inhalation of intact cotton dust. Furthermore, the sample sizes for these studies was small and the outcome may have been due to random variability. A specific study with the appropriate number of subjects is needed to determine the relationship of non-specific bronchial reactivity to responsiveness to inhalation of aerosols of cotton dust.

Some occupational exposures have been shown to produce an increase in non-specific bronchial reactivity as well as sensitization to the agent itself (23). However, increased bronchial reactivity has also been shown to occur after inhalation of air pollutants which are not sensitizes (24). A recent study reported that non-specific bronchial reactivity increased after the workshift in textile workers with mild symptoms of byssinosis (25). Unfortunately the pre and post-shift studies of bronchial reactivity were carried out a week apart and thus may have been influenced by factors other than the dust exposure on the day of the second, that is, post-shift study. More recently Boehlecke has demonstrated an increase in non-specific bronchial reactivity in naive subjects exposed to aerosols of cotton dust (26). It is important to attempt to establish whether cotton dust inhalation can induce non-specific bronchial hyperactivity and to determine if there are differences in the response of atopic and non-atopic individuals. This has implications for

possible mechanisms of interaction between multiple exposures (e.g., cigarette smoke and cotton dust) as well as for research to identify those at greater risk of chronic loss in function. Studies of relatives of patients with chronic obstructive lung disease have indicated that those with non-specific bronchial hyperactivity have a more rapid rate of annual decline in lung function than those without hyperactivity (27).

Investigations of textile workers have not found evidence that the response to cotton dust is immunologically medicated; the workers with symptoms or functional changes are not sensitized or "allergic" to cotton dust (15). This was confirmed in a recent epidemiology study of the U.S. cotton textile industry (17). This study reported that specific sensitization to extracts of cotton dust as manifest by skin testing did not correlate with the magnitude of overshift decline in FEV_1. This study did show that in the early processing of cotton (the opening and carding areas) workers with two or more positive skin prick responses to a battery of common allergens and with a personal or family history of atopy had a significantly greater decline in FEV_1 to cotton dust than non-atopic workers. Approximately 10 % of the workers in this study were classified as atopic (by skin test); a figure that is in agreement with atopy in the general population. Confirmation that the percent responding and magnitude of response is larger for atopics than non-atopics would improve our ability to characterize the response to cotton dust and better identify those at risk.

Numerous reports have documented an influx of neutrophils into the airways after exposure to cotton dust and various constituents of cotton dust (28). The increase in neutrophils has been stated to correlate with the ventilatory function response and studies of workers exposed to cotton dust have shown increased numbers of neutrophils collected by nasal swabs (4). The cell population in the lung associated with the airway epithelium may be an indicator of airways reactivity and therefore associated with the magnitude of the acute response to cotton dust. Nasal lavage is a non-traumatic technique to characterize the cell population on the epithelial surface of the nose and appears to correlate closely with the cell population on the surface of the lower airways (29,30). Nasal lavage will provide a means to characterize the airways cell population and to determine if changes in nasal neutrophils correlates with physiologic responses to the exposure.

There is increasing evidence that many of the clinical effects observed among persons exposed to cotton or other organic dust are related to the presence of acute or chronic inflammation in the lung tissue rather than IgE-mediated activity or a bronchoconstriction induced by mediators acting on smooth muscle (31). A key cell associated with the development of reactions occurring after the inhalation of different agents is the lung macrophage. Its precursor, the blood monocyte, exhibits reactions which have been related to pulmonary disease. Mononuclear cells accumulate at a site of chronic infection as well as in lung tissue after exposure to organic dust in humans and in animal models used to assess exposure to cotton dust (32,33). The blood monocyte and the pulmonary macrophage can be induced to produce procoagulant activity (34). This reaction can be mediated by specific antigens (35) and is related to delayed type hypersensitivity reactions (36), which are now understood to reflect cell-mediated immune responses (37). A relationship between the blood coagulation sequence and the appearance of delayed type hypersensitivity has been demonstrated (36). In other studies, in which the blood

coagulation was influenced by anti-coagulant treatment, it was found that the skin response to allergens in experimental animals disappeared (38,39). In addition to the skin reactions previously studied , evidence from investigations on persons exposed to airborne organic dust demonstrate the presence of increased number of lymphocytes in the airways (40). In view of these studies the responsiveness of peripheral blood monocytes (as measured by procoagulant activity on stimulation with a mitogen) may be a predictor of responsiveness to cotton dust.

Thus, the question of the relationship of pre-existing atopy and/or bronchial hyperactivity to the responsiveness to cotton dust is not fully answered. Therefore these studies sought to characterize the response of subjects pre-screened for atopic status and non-specific bronchial hyperactivity to aerosols of cotton dust.

1990 Study

Introduction

In 1990, the study was conducted at the Department of Environmental Medicine at the University of Gothenburg, Gothenburg, Sweden. In addition to Dr. Jacobs from the University of Alabama at Birmingham (UAB) co-investigators included Dr. Brian Boehlecke from the University of North Carolina (UNC) and Dr. Ragnar Rylander from the University of Gothenburg. This study specifically sought to characterize the ventilatory and methacholine response of naive subjects, categorized by atopic status, to a single 4 hour exposure to an aerosol of cotton dust. Additionally, pre and post exposure nasal lavage and blood samples were collected for each subject. Cell numbers were evaluated in the nasal lavage samples and cell type and reactivity were evaluated for the blood samples.

Results

The results of these studies are described in the papers in Appendix One. The following points summarize the results of the study:

1. There was good agreement between atopic status determined by questionnaire and by the Phadiatop, an objective method of determining atopic status which evaluates the levels of serum IgE antibodies to a series of relevant inhalant allergens.

2. Atopic subjects had a significantly greater mean fall in FEV_1 than non-atopics (8.3% vs 4.9% $p<0.001$) confirming that atopics do exhibit a greater ventilatory response to cotton dust than do non-atopics, however, both groups responded to the dust.

3. Atopic subjects had a significantly higher baseline methacholine responsiveness than did non-atopic subjects (26% vs 0% reaching a PD_{20}, $p<0.0005$) indicating that atopics may be more responsive to various environmental exposures than non-atopics.

4. After cotton dust exposure, there was a significant increase in airway reactivity in both atopics and non-atopics (68% vs 20% reaching a PD_{20}, $p<0.0005$). These data suggest that although atopics are more responsive than non-atopics, cotton dust increases airway reactivity in both atopic and non-atopic subjects. One may speculate that at high dust levels, atopics will be severely affected and will not continue to work in those environments (healthy worker effect) and non-atopics will exhibit a mild ventilatory decrease and increased airway reactivity. At low dust levels, atopics may not select themselves out of the environment and will respond with both an ventilatory decrease and increased airway reactivity while non-atopics will show a minimal ventilatory and airway reactivity response.

5. Baseline nasal lavage studies indicated that the mean pre-exposure cell count was higher in atopics (2.7×10^4 cells/ml) than non-atopics (1.4×10^4 cells/ml), however these differences were not significant. The elevated baseline data suggest that a chronic state of

inflammation may exist in the upper respiratory tract of atopics.

6. Pre to post exposure cell numbers in nasal lavage increased significantly for all subjects after exposure to cotton dust confirming that cotton dust causes an acute inflammatory response and suggesting that nasal lavage may be a technique that can be used to model the effects that inhaled inflammatory agents have more distally in the lung. When the pre to post-exposure cell lavage data were sorted by atopic status, the significance of the increase in cell numbers after exposure persisted only for the non-atopic subjects with the lower pre-exposure lavage counts. These data suggest that an elevated pre-exposure inflammatory state may indicate an increased risk for an acute ventilatory response to cotton dust. Those subjects with the higher pre-exposure nasal lavage cell counts had larger drops in ventilatory parameters.

7. Cotton dust induced ventilatory responses were related to the preexposure PCA production in blood monocytes after stimulation by lipopolysaccharide. No relationship was observed between PCA production and atopic status nor was there a predictable change in PCA production in blood monocytes collected after exposure to cotton dust. The association of increased PCA production in blood monocytes collected prior to exposure and increased ventilatory responsiveness may be related to a genetic or acquired disposition of blood monocytes to respond. However, no comment can be made at this time regarding the significance of this finding, and further studies are needed to support this hypothesis.

8. Assessment of the blood cell parameters also suggest that selected subsets of T lymphocytes were elevated in atopic subjects. Significantly higher levels of $CD8+S6F1^{lo}$ cells and a higher ratio of $CD8+S6F1^{lo}/CD8+S6F1^{hi}$ cells occurred in atopic subjects. These data suggest that ventilatory changes induced by an inflammatory response in the airways may be related to pre-existing cell types and cell responsiveness; however, at this time no unifying hypotheses is proposed to account for this association.

9. Tables 1 and 2 show the symptoms immediately post-exposure and at 24-hours post-exposure for physician-confirmed atopics and non-atopics. Specific symptoms included dry cough, cough/phlegm, chest whistling, chest tightness, difficulty breathing, shortness of breath, fever, and flu-like symptoms. Immediately post-exposure, both atopics and non-atopics reported most frequently the symptoms for dry cough, cough with phlegm, and chest tightness. These symptoms occurred more frequently and were more severe in atopics. Both atopics and non-atopics reported fewer symptoms for difficulty breathing, shortness of breath, fever, or flu-like symptoms, and non-atopics reported fewer symptoms than atopics for chest whistling.

   At 24-hours post-exposure, both atopics and non-atopics reported more categories of symptoms as well as an increase in symptoms reported as moderate. There appeared to be no differences in the percent or types of symptoms between atopics and non-atopics 24-hours post-exposure.

Presentations

1. Beijer L., Rylander R., Boehlecke B. and Jacobs R. (1991). Blood Monocyte Procoagulant Activity As A Predictor of Organic Dust Induced Pulmonary Function Decrease. American Thoracic Society Meetings.

2. Boehlecke B., Jacobs, R. and R. Rylander (1991). Association of Mild Atopy and Airway Response To Cotton Dust. Presented at the Fourth International Conference on Environmental Lung Disease. Sept. 25-28 Montreal, Quebec, Canada.

3. Rylander, R. and R. Jacobs: Bronchial Reactivity After Cotton Dust Exposure. International Workshop on Byssinosis, December 8-9, 1993, Guargzhaw, The People's Republic of China.

Publications

1. Jacobs, R.; Boehlecke, B.; van Hage-Hamsten, M.; and Rylander, R.: Bronchial Reactivity, Atopy and Airway Responses to Cotton Dust. American Review of Respiratory Disease 148:19-24, 1993.

2. Beijer, L.; Jacobs, R.; Tengborn, L.; Andersson, B.; and Rylander, R.: Cell Reactivity Predicts Cotton Dust-Induced Bronchoconstriction. 1995. (Submitted: American Review of Respiratory Disease).

3. Jacobs, R.; Boehlecke, B.; and Rylander, R.: Cellular In flux in Nasal Lavage of Humans Exposed to Cotton Dust, In: Proceedings of the 16th Cotton Dust Research Conference, Eds. L.N. Domelsmith, R.R. Jacobs, and P.J. Wakelyn, New Orleans, La., National Cotton Council, Memphis, Tn., pp:215-218, 1992.

Tables

Table 1 The Response of Atopics and Non-Atopics to the Symptoms Questionnaire Immediately Post-Exposure

	Non-Atopics (n = 32)				
	No Response (0)	Mild (n/%)	Moderate (n/%)	Severe (n)	% of Subjects Responding (n/%)
Dry Cough	12	17/53	3/9	0	20/63
Cough/Phlegm	26	5/16	1/3	0	6/19
Chest Whistling	31	1/3	0	0	1/3
Chest Tightness	22	7/22	3/9	0	10/31
Difficulty Breathing	32	0	0	0	0
Shortness of Breath	32	0	0	0	0
Fever	32	0	0	0	0
Flu-Like Symptoms	31	1/3	0	0	1/3
	Atopic (n=20)				
Dry Cough	4	12/60	3/15	1	15/80
Cough/Phlegm	14	6/30	0	0	6/30
Chest Whistling	15	3/15	2/10	0	5/25
Chest Tightness	11	5/25	3/15	1	8/45
Difficulty Breathing	20	0	0	0	0
Shortness of Breath	18	2/10	0	0	2/10
Fever	20	0	0	0	0
Flu-Like Symptoms	19	0	0	0	0

Table 2 The Response to the Symptoms Questionnaire 24 Hours Post-Exposure

	Non-Atopic (n=32)				
	No Response (0)	Mild (n/%)	Moderate (n/%)	Severe (n)	% of Subjects Responding (n/%)
Dry Cough	19	9/28	4/13	0	13/41
Cough/Phlegm	24	6/19	2/6	0	8/25
Chest Whistling	30	1/3	1/3	0	2/6
Chest Tightness	17	10/31	5/16	0	15/47
Difficulty Breathing	31	1/3	0	0	1/3
Shortness of Breath	25	7/22	0	0	7/22
Fever	21	6/19	5/16	0	11/24
Flu-Like Symptoms	29	11/30	0	0	11/30
	Atopics (n=18)				
Dry Cough	9	7/35	2/6	0	9/45
Cough/Phlegm	15	2/10	1/5	0	3/15
Chest Whistling	16	1/5	1/5	0	2/10
Chest Tightness	13	2/10	3/15	0	5/25
Difficulty Breathing	16	2/10	0	0	2/10
Shortness of Breath	13	3/15	2/10	0	5/25
Fever	15	3/15	0	0	3/15
Flu-Like Symptoms	15	3/15	0	0	3/15

1991 Study

Introduction

Results from the studies in 1990 confirmed that naive subjects, defined as atopic, respond with a significantly larger acute change in FEV_1 than non-atopic subjects. Both atopic and non-atopic subjects also had increased non-specific bronchial hyper-reactivity after cotton dust exposure. The increase for atopics was larger than the increase for non-atopics. Although both groups responded to aerosols of cotton dust, these data indicate that individuals that are atopic are at greater risk for an acute response to cotton dust than non-atopic subjects. Based on data from these studies the following questions were addressed in 1991.

1. Is the acute overshift response as measured by FEV_1 a meaningful predictor for chronic impairment? There are some studies that suggest the acute overshift declines in FEV_1 from exposure to cotton dust is a predictor of annual change in FEV_1 (51,56) however, the risk is not well defined. Previous studies have shown that for other inhaled substances increased non-specific bronchial reactivity is related to an increased risk for the development of chronic obstructive lung disease (24). If repeated exposures to aerosols of cotton dust increase non-specific bronchial responsiveness then those workers may be at greater risk for developing COPD.

2. Do cottons with variable levels of endotoxins cause a dose dependent response in non-specific bronchial responsiveness? Studies to evaluate this question can aid in determining the role of endotoxin in the long term response to cotton dust. These studies can be extended to other constituents of cotton, such as tannins, by identifying cottons with variable levels of each specific constituent.

3. Do specific treatments of cotton, such as washing to remove endotoxin, remove or reduce the response to cotton dust measured by other markers such as non-specific bronchial hyperresponsiveness? Previous data have show than the acute pulmonary response to cotton dust (FEV_1) is reduced by washing, however no other markers of responsiveness were evaluated by these studies.

4. Is the response of atopics to aerosols of cotton dust containing variable levels of endotoxin (or other constituents) different than the response of non-atopics to the same dust?

Methods

Studies for 1991 were done in the model cardroom at the ARS facility in Clemson, SC. Lifetime non-smokers without a history of asthma or previous exposure to cotton dust were recruited by radio and newspaper advertisement, and invited to a follow-up screening session at the laboratory. Laboratory screening consisted of spirometry, administration of a more detailed medical and occupational questionnaire, and if not excluded by questionnaire response, collection of a sample of venous blood for evaluation of atopic status. The questionnaire asked

about personal and family history of allergy and previous exposure in dusty environments.

Subjects selected for the study were asked to participate two consecutive days a week for five weeks. Each week participants reported the day prior to dust exposure for methacholine bronchoprovocation testing. The next day, prior to entering the cardroom, participants completed baseline spirometry. Each participant remained in the cardroom for 5 hours during which lunch was provided; restroom breaks were allowed as needed. Subjects were removed from the cardroom after 5 hours and spirometry and methacholine testing performed. A symptom questionnaire was completed immediately post-exposure and again at 24 hours. The same pre-test and exposure day was maintained for each subject during the five week study.

During the first four weeks each group was exposed, in a Latin square design, to dust from one of the four types of fiber described below. This resulted in all participants being exposed to each condition by the end of the study. During the fifth week all subjects were exposed on their specific day to aerosols of the Texas washed cotton at 0.5 mg/m^3.

Dust was generated from four types of fiber: rayon, California and Texas unwashed cottons and the Texas cotton after washing. The target dust level for each cotton was 1 mg/m^3. The target dust level for rayon was 0.5 mg/m^3 which was the maximum achievable for the carding conditions and minimum room ventilation criteria. Ambient air was sampled for total viable airborne bacteria with six-stage Anderson air samplers loaded with 90 mm plastic petri plates filled with 40 ml of trypticase soy agar [30 g trypticase soy broth, 20 g agar and 5 g yeast extract per liter and cycloheximide (50 μg/ml)]. The plates were incubated for 22-30 hours at room temperature (RT;24.1±03°C). Airborne endotoxin was evaluated by extracting VE filters in 50 ml pyrogen free water (PFW) for 1 hour and assayed using the *Limulus* amebocyte lysate (LAL) assay (BioWhittaker).

Results

The results of these studies are given in the papers in Appendix Two and summarized below.

1. **Study Population**: A total of 38 subjects participated in the study. The demographic data for the subjects are shown in Table 1. Fifteen subjects were categorized as atopic by the Phadiatop assay for IgE antibodies to specific common aeroallergens. The Phadiatop was later confirmed with a second Rast test (Ventrex) and there was no difference in subjects identified as atopic by either Rast test. A larger portion of the non-atopic subjects were female (87 vs 67%) and non-atopics were on average 3.5 years older than atopics. Although atopics had slightly higher baseline values for FEV_1 and FVC as percent of predicted than the non-atopic subjects, these differences were not statistically significant.

2. **Screening Questionnaire:** Eighteen of 38 subjects gave a personal history of atopy on questionnaire and 16 reported this had been confirmed by a physician. However, only eight of the these subjects (44%) were classified as atopic by the Phadiatop. Of the subjects classified as atopic by the Phadiatop 30% gave an negative history of atopy (Figure 1). These data differ from those observed in year one of the study. In 1990, the

positive predictive value (PPV) was 100% and the negative predictive value (NPV) was 84%. PPV referred to the proportion of subjects who said they were atopic by questionnaire and who were atopic by objective measure. NPV referred to the proportion of those subjects who said they were not atopic and were not atopic by objective measure. In 1991 the PPV for atopy was 44% and the NPV was 65%. These data would suggest there was a difference in the accuracy of the response of these subjects to the questionnaire from that of Swedish subjects. The latter appeared to be better informed regarding their atopic status than subjects recruited for the study at Clemson, SC. Two variables may in part account for the differences in the questionnaire response: the differences in the medical system that is in place in Sweden and the United States and the socio-economic status of the subjects in the two studies. In Sweden, essentially all of the subjects were students which represents a higher socio-economic group than other populations in Sweden. At Clemson, the study population consisted of a heterogeneous group which included not only students but also the unemployed and retired subjects. At the time of this study the medical system in Sweden was highly socialized and all medical cost covered by the government. It is more likely that a Swedish citizen would request a physician evaluation for mild symptoms. If a physician were to suspect that a patient were atopic and recommend an objective test be done to confirm their atopic status the cost would not deter having such a test done. However, under the medical system in place in the United States, a physician might indicate that a patient is atopic, but a recommendation that their atopic status be confirmed by an objective measure would be dependent on the ability and desire of the patient to pay for the test or the insurance company to pay for the test. Other variables, as yet unaccounted for, are likely to impact on the results from these studies; however, questionnaire responses appear not to be a reliable means for determining atopic status.

3. **Symptom Questionnaire Response**: Subjects were asked to respond to a questionnaire regarding symptoms immediately after each exposure and at 24 hours. Questions pertaining to the severity of cough, phlegm, chest tightness (CT), wheeze, shortness of breath (SOB) and difficulty in breathing (DB) were ranked as none, mild, moderate, and severe. Table 2 shows the number and percentage of subjects reporting at least one symptom. The number of subjects reporting at least one symptom was greatest for Texas unwashed cotton (64%, immediately post-exposure and 50%, 24 hours post-exposure) followed by Texas washed cotton at one mg dust (46% and 53%, respectively). Fewer subjects reported symptoms from exposure to either the rayon or California cotton. In general, except for the Texas unwashed cotton, symptoms were reported more frequently at 24 hours post-exposure (Figure 2). For all exposures, a greater proportion of atopic subjects reported symptoms than non-atopics (Figure 3, Table 2). However, the differences were not significant.

   Testing differences between proportions demonstrated that significantly more subjects (both atopic and non-atopic) reported at least one symptom when exposed to Texas unwashed cotton (Table 2). More atopic subjects reported symptoms than non-atopic subjects; however, the differences were not significant. Immediately post-exposure, significantly fewer subjects (overall and atopic/non-atopic) reported symptoms resulting

from California and rayon exposures. For Texas washed, the symptom response was mixed. The proportion of non-atopic subjects reporting at least one symptom immediately after exposure to washed cotton was not significantly different from unwashed cotton. The proportion of atopic subjects reporting symptoms immediately after exposure to washed cotton did not differ from that after either unwashed cotton, California cotton, or rayon. At 24 hours post-exposure, there was no significant difference in the number of subjects reporting symptoms for any of the exposures.

The response to the questionnaire showing individual symptoms is shown in Table 3 and Figure 4. Cough, phlegm, and chest tightness were the symptoms reported most frequently. The majority of subjects reported that the symptoms experienced were mild. Since few subjects reported moderate or severe symptoms, no analysis was done regarding symptom severity.

4. **Exposure Conditions**: The mean VE dust concentrations for the four exposure days for each condition, are shown in Table 4. The dust level for rayon was approximately one-half the level for the cotton exposures except for week five. The rayon concentration ranged from 0.41 to 0.50 mg/m^3 with 64% of the particles less than 4.4 μm in aerodynamic diameter. For California, Texas unwashed, and the Texas washed cotton the dust levels were maintained at approximately 1 mg/m^3. During the fifth week all subjects were exposed to washed cotton at 0.5 mg/m^3. For each cotton exposure, the range over the four days was less than ± 8% of the individual means. For California cotton 61% of the particles had a mean aerodynamic diameter <4.4 μm and for Texas unwashed cotton 73% of the particles were <4.4 μm. For the Texas washed at both 1 and 0.5 mg/m^3, 58% of the particles were <4.4 μm indicating a shift towards larger particles for the washed cotton.

Airborne total bacterial counts were highest for Texas unwashed and lowest for rayon (Table 4). There was no significant difference between the levels of airborne bacteria for the rayon and California exposures. Airborne bacteria levels generated from the Texas washed cotton at 1 mg/m^3 were significantly lower than for the unwashed cotton ($P < 0.05$). Reduction of the dust level of washed cotton to 0.5 mg/m^3 reduced the airborne bacterial levels by approximately 50%. The airborne bacterial levels in dust generated from washed cotton at both 1 and 0.5 mg/m^3 were significantly higher that those for either the California or rayon exposures ($p < 0.05$). The bacterial levels for both California and rayon were similar to the levels measured in the cardroom with no fiber being processed at normal exposure room ventilation conditions. Statistical comparison between control room levels and the different exposures could not be done because control room conditions were measured on only one day. The control room levels are similar to those reported in previous studies in this chamber.

Endotoxins in vertical elutriator lint and airborne dust levels are shown in Table 5. For the lint, rayon had the lowest value (2.3 Eu/mg) followed by Texas washed (72 EU/mg), California (82 Eu/mg) and Texas unwashed (260 Eu/mg). The magnitude of the differences in the endotoxin on the lint was reflected in the endotoxin in vertical elutriator

samples. There was no significant difference in the levels of vertical elutriator dust endotoxin between the rayon exposure, the California exposure or the Texas washed exposure at 1.0 mg/m^3. Airborne vertical elutriator endotoxin for Texas washed at 0.5 mg/m^3 were not measured but was assumed to be one-half of the airborne levels of the Texas washed at 1 mg/m^3. The vertical elutriator levels of airborne endotoxin associated with the unwashed Texas cotton was significantly higher than any of the other exposures.

5. **Pre-Exposure Ventilatory Function**: Experimental exposures were done using a Latin square design in which each group was exposed over a four week period to a different condition. Prior to each week's exposure a pre-spirometry and pre-methacholine evaluation was done. The pre-exposure data were analyzed to determine if there was a trend in baseline data that may reflect differences in atopic and non-atopic subjects or reflect a carry-over from the exposure of the previous week. Table 6 shows the summary pre-test FEV_1 and FVC data (as percent of predicted) for each week of the four-week study. These data were analyzed using a General Linear Model ANOVA in which week, atopic status, and exposure were dependent variables (Table 7). Atopic status was not a significant term in the model ($P = 0.2$). The model indicated there was an effect of week over the four-week study for both pre-test FEV_1 and FVC ($p < 0.0001$). For both FEV_1 and FVC, baseline pre-exposure values were significantly higher on week one of the study than for the remaining three weeks of the study. Exposure was shown to be a borderline significant term for FEV_1 ($p=0.046$), but not for FVC or the FEV_1/FVC ratio. These data suggest there was an effect of fatigue or negative learning over the four week study.

   Although, atopic status was not a significant term in the model, the data for atopics and non-atopics were evaluated separately to determine if there were trends in the data (Tables 8 and 9). Evaluation of the non-atopic data alone (Table 8) confirmed the effect of week and exposure for both FEV_1 and FVC. Week one pre-test values were significantly higher than weeks two through four for both FEV_1 and FVC. For atopics alone, there was no effect of either week or exposure (Table 9).

6. **Pre-exposure Methacholine Responsiveness:** Summary data for pre-exposure methacholine responsiveness are shown in Table 10. Methacholine responsiveness is reported as the slope of a line derived from a least-squares regression of the change in FEV_1 from the saline value against the log cumulative dose of methacholine. Input of these data into the model showed no effect of week, although for atopics there was a tendency of lower responsiveness for week one than for the remaining weeks of the study.

   Evaluation of these data using the statistical model indicated that atopic status was a significant variable ($p = 0.04$, Table 11). Overall, the mean pre-exposure slopes were all significantly different from zero and as a group, there was a non-significant tendency for increased responsiveness over the four-week study.

   Table 12 compares the mean slope for atopics to that of non-atopics for the four-week study. Atopics had a significantly larger slope than non-atopics. These data indicate that

atopics had a greater baseline methacholine responsiveness. For non-atopics, the mean pre-exposure slope was not significantly different from zero; however, for atopics, the slope was different. The pre-exposure slope of atopics was approximately three times the slope of non-atopics. These data are similar to the results reported in 1990. The data for atopics and non-atopics by week are shown in Table 13. There was a significant difference in methacholine responsiveness between atopics and non-atopics for each week of the study. There was no trend over the four-week study for either group, however, atopics tended to be less responsive in week one.

7. **Exposure Related Changes in Spirometry**: Table 14 shows the summary data for the FEV_1, FVC, and FEV_1/FVC ratio for each of the five exposures done during the study. The data for FEV_1 and FVC are expressed as the absolute difference in liters and the data for the ratio are expressed as the absolute difference in the pre and post-exposure ratio. Positive values indicate the pre-exposure value was higher than the post-exposure value, i.e. ventilatory function decreased during the exposure. These data, evaluated using the GLM Anova (Table 15), demonstrated a significant effect of exposure on FEV_1, FVC, and the Ratio ($p < 0.001$, $p < 0.003$, and $p < 0.05$, respectively). Atopic status was not a significant term in the model ($P > 0.2$). There was no difference in the response of the subjects to California cotton, the washed Texas cotton at 1 mg/m^3, and rayon. The response to the Texas unwashed cotton was significantly larger than the response to the other cottons. This trend was consistent for FEV_1, FVC, and the FEV_1/FVC ratio. For all exposures, the absolute change (in liters) for FEV_1 was significantly different from zero, indicating that there was a biological response to all the dust that was different from no change. For FVC, the absolute change (in liters) was significantly different from zero for only the Texas washed and unwashed cotton. A similar response to that observed for the FVC was observed for the ratio. These data would suggest that the response was consistent with a pattern of airway obstruction for the Texas unwashed cotton.

Although atopy was not a significant variable in the model, the data were evaluated as an overall response (Table 16). Atopics were slightly more responsive than non-atopics for FEV_1 and FVC, but the differences were not significant.

The statistical model used for this study assumes that the variance is constant for each week or exposure condition and used a pooled estimate for all comparisons. Therefore, the standard error estimates are similar for each exposure. The question was asked, is the use of common variance valid if one of the exposures causes a change in FEV_1 that much larger than that caused by the other exposures? To determine if the large changes from the exposure to Texas unwashed cotton affected the comparison of the other exposures, unwashed cotton was removed from the model. Results of exposure to rayon, California, and Texas washed cotton are shown in Table 17. None of the exposures caused a change in FEV_1, FVC, or the ratio that was significantly different from another, although there was a non-significant trend in which the washed cotton response was larger than either rayon or the California cotton.

Although atopy was not a significant variable in the model, the data were evaluated

separately (Table 18). As previously shown, a trend of increased ventilatory responsiveness was seen in atopics for all cottons. Further analysis of the data for atopics and non-atopics separately confirmed there was no difference in the ventilatory response to the California cotton, the Texas washed cotton at 1 mg dust and the rayon (Tables 19 and 20).

8. **Exposure Related Changes in Methacholine Responsiveness:** All exposures produced an increase in airway responsiveness to methacholine indicated by an increase in the slope of the fitted FEV_1 - methacholine dose response curve (Table 21). The increases in responsiveness after exposure to Texas unwashed cotton and Texas washed cotton were significantly greater than those after the other two exposures. The increase in responsiveness after Texas washed cotton was not different from that after Texas unwashed despite the approximately 10 fold lower airborne endotoxin concentration in the former. The change in responsiveness after washed cotton was significantly greater than the response to California cotton although the latter had a slightly greater endotoxin content. There was a tendency for atopics to show greater increases in responsiveness than non-atopics, but this reached significance only for the exposure to California cotton. For both groups the Texas cotton and Texas unwashed cotton produced significantly greater increases in methacholine responsiveness than California cotton or rayon but were not different from each other despite the large difference in endotoxin content.

   A separate exposure to 0.5 mg/m^3 of Texas washed cotton, not part of the Latin square design, was done on all participants in week five. The group mean fall in FEV_1 was 40 ml which was not significantly different from that after the exposure to California cotton at 1 mg/m^3 or rayon at 0.5 mg/m^3. There was also an increase in airway responsiveness to methacholine which was not different from that present after the California cotton despite the approximately four-fold lower airborne endotoxin concentration during the washed cotton exposure.

Summary

Results from the studies in 1991 demonstrated that both atopic and non-atopic subjects exposed to aerosols of cotton dust with different levels of endotoxins increased non-specific bronchial hyperresponsiveness as measured by methacholine challenge. Atopics tended to have greater drops in FEV_1 and increases in methacholine responsiveness, however, the differences were generally not significant. Although the changes were small, both atopic and non-atopic subjects demonstrated increased airway reactivity and acute pre to post-exposure changes in FEV_1 to aerosols of dust from rayon. These data indicate that rayon is not a completely inert dust. The responses to California cotton were not significantly different from rayon. Washing cotton did remove the acute ventilatory response (FEV_1) relative to exposure to rayon although there was still a trend of higher change in FEV_1 response to washed cotton. However (a significantly higher increase in the level of methacholine responsiveness) occurred in the subjects after exposure to washed cotton at 1 mg/m^3 compared to rayon. When the exposure level of washed cotton was reduced to 0.5 mg/m^3, both the acute ventilatory response (change in FEV_1) and change in methacholine responsiveness were no different that those seen with rayon exposure.

The increased responsiveness to methacholine for the four types of exposure did not correlate with the endotoxin levels in the dust. The acute ventilatory response did correlate with the endotoxin level. These data would suggest either that physiologic mechanism resulting in increased bronchial reactivity is different than the mechanism causing a change in FEV_1 and that washing fails to remove all the agents causing increased bronchial hyperresponsiveness, or that bronchial reactivity is a more sensitive indicator of the acute response to cotton dust.

Presentations:

1. Harder S., Boehlecke B., Jacobs R., Becker S, and Devlin R. Spirometric and Inflammatory Response of Humans Exposed to Cotton Dust Containing Different Amounts of LPS. American Thoracic Society Meetings, 1993.

2. Boehlecke BA, Jacobs RR, and Perkins H (1994). 5 Hour Exposures to Cotton and Synthetic Fiber Dusts Cause Increases in Airway Responsiveness in Healthy Volunteers. Presented at the Fifth International Conference on Environmental Lung Disease. March 2-5, 1995 Orlando,Fla.

3. McCann, P. and R.R. Jacobs: Comparison of Methods for Measuring Endotoxin in Organic Dust. Presented at the American Industrial Hygiene Conference and Exposition, 1992.

Publications

1. Boehlecke BA, Jacobs RR, Perkins L, and Perkins H. Inhaled Particulates Cause Increased Airway Reactivity Not Explained by Endotoxin. (Draft manuscript in appendix).

2. Jacobs, R.; Boehlecke, B.; Perkins, H.H.; and Chun, D.: Evaluation of the Acute Pulmonary Response to Aerosols of Dust From Batch-Kier Washed Cotton. In: Proceedings of the 17th Cotton Dust Research Conference, pp:274-278, 1993. Eds. L.N. Domelsmith, R.R. Jacobs, and P.J. Wakelyn, New Orleans, La., National Cotton Council, Memphis, Tn.

3. Jacobs, R. and Atchley, K.: An Evaluation of Available Methods for Measuring Condensed Tannins in Airborne Samples of Vertical Elutriated Cotton Dust. In: Proceedings of the 17th Cotton Dust Research Conference, pp:329-331, 1993. Eds. L.N. Domelsmith, R.R. Jacobs, and P.J. Wakelyn, New Orleans, La., National Cotton Council, Memphis, Tn.

Tables and Figures

Figure 1. Comparison of the Phadiatop Rast Results to the Questionnaire Response Results for Determination of Atopic Status

	Phadiatop		
Hayfever	+	-	Total
+	8	10	18
-	7	13	20
Total	15	23	38

Figure 2 Percentage of Subjects Reporting at Least One Symptom

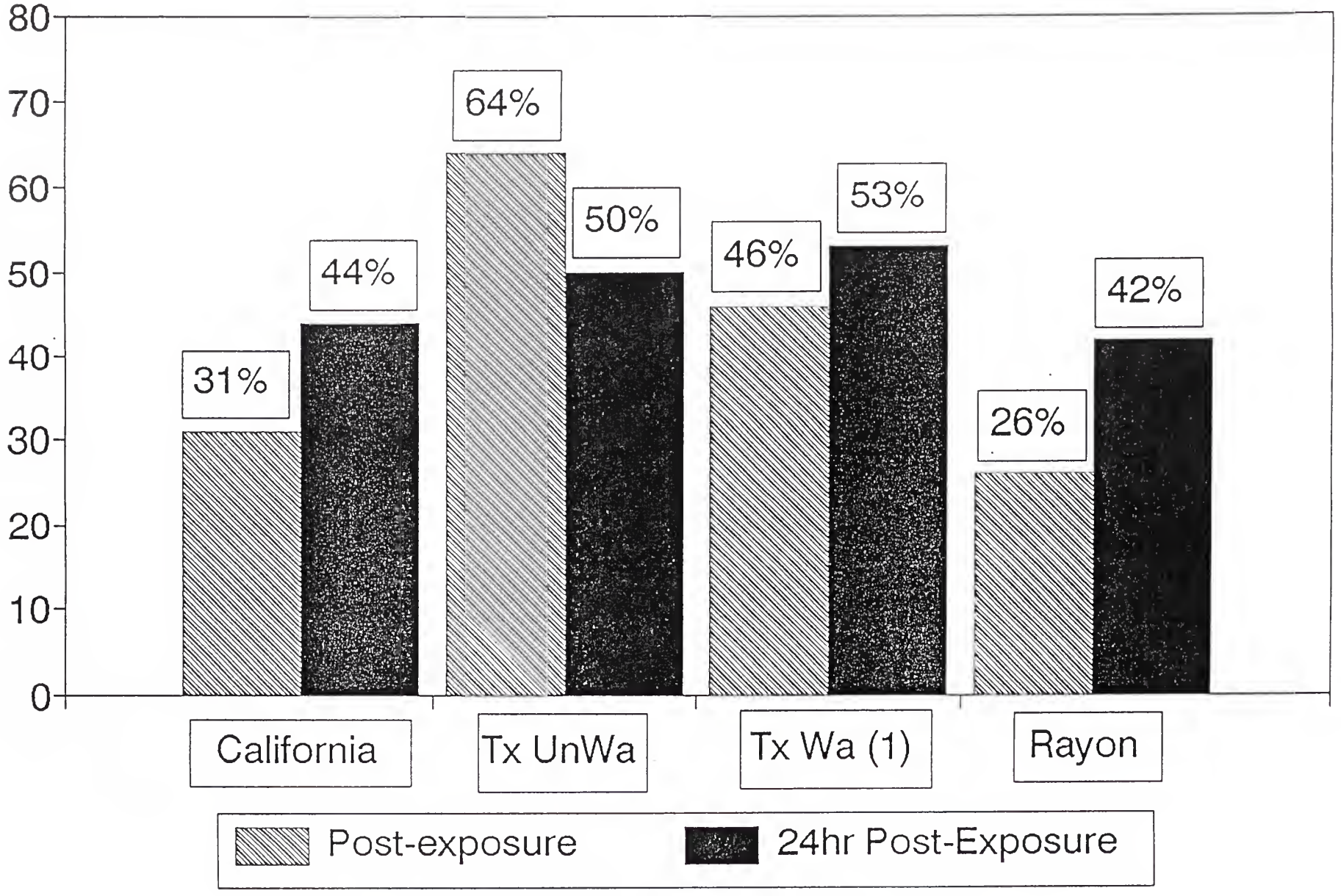

Figure 3 Percentages of Subjects Reporting at Least One Symptom

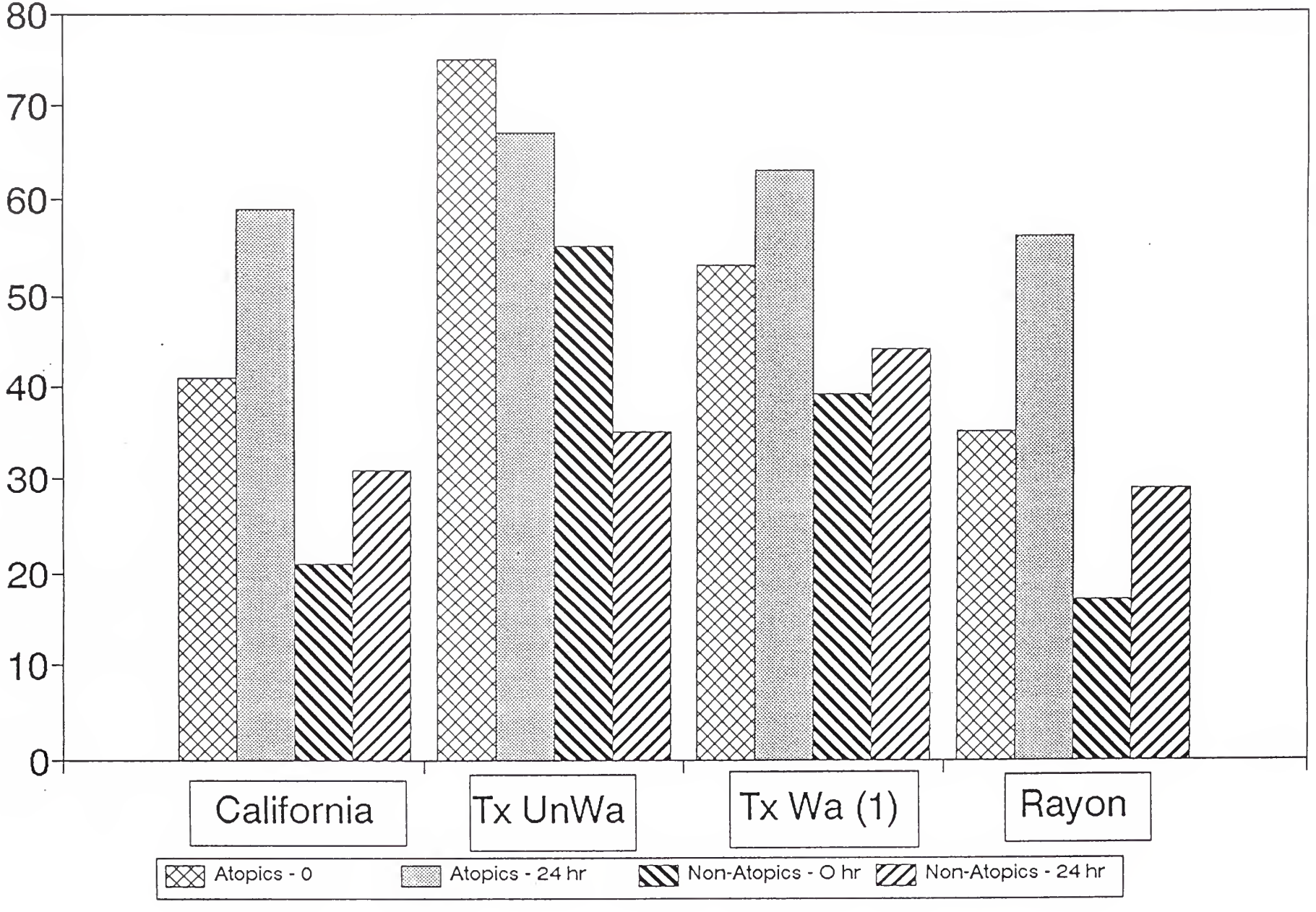

Figure 4 Percent of Subjects Reporting Symptoms

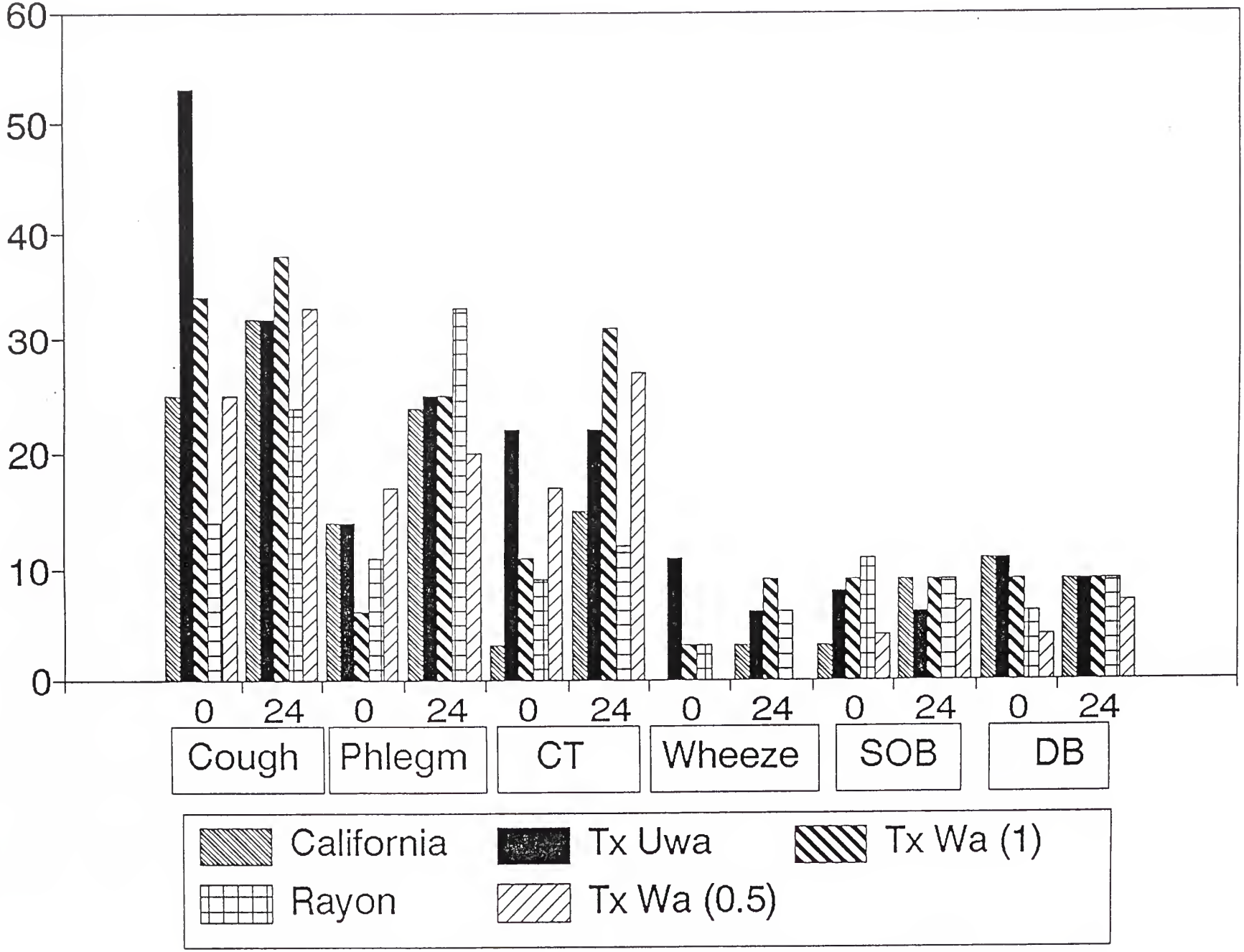

Table 1. Descriptive Characteristics of the Study Population

	Atopics[1]	Non-Atopics	Total
Number of subjects	15	23	38
MALE %	33.3	13.0	21.0
RACE (% white)	87	83	84
AGE	31.0 (8.3)	34.5 (12.0)	33.1 (10.8)
% History of Hayfever	53.3	45.3	-
MD confirmed Hayfever	7/8	9/10	
FEV_1% Pred.	106.2 (12.3)	101.8 (9.9)	103.6 (11.0)
FVC% Pred.	106.8 (13.2)	101.6 (10.4)	103.6 (11.7)
FEV_1/FVC (%)	82.5 (1.8)	83.4 (3.1)	83.0 (2.7)
Mean Phadiatop (% Binding)	10,548[2] (6037)	678 (122)	4574.0 (6137.6)
Ventrex - Seasonal	5824.9[2] (6063.2)	279.5 (180)	2468.5 (4677.4)
Ventrex - Annual	11,738.4[2] (6427.4)	342.2 (200)	4840.7 (6881.9)

[1] By Phadiatop

[2] Significantly different from non-atopics ($p>0.05$)

Standard deviation in parenthesis

Table 2. Number and Percentage of Subjects Reporting at Least One Symptom By Questionnaire

		Immediately Post-Exposure			24-hrs Post-Exposure		
		Overall	Atopic	Non-Atopic	Overall	Atopic	Non-Atopic
California	n	11/36[a]	7/17[a]	4/19[a]	15/34[a]	10/17[a]	5/16[a]
	%	31	41	21	44	59	31
Tx Unwashed	n	23/36[b]	12/16[b]	11/20[b]	16/32[a]	10/15[a]	6/17[a]
	%	64	75	55	50	67	35
Tx Washed	n	11/35[a]	9/17[a,b]	7/18[b]	17/32[a]	10/16[a]	7/16[a]
	%	46	53	39	53	63	44
Rayon	n	9/35[a]	6/17[a]	3/18[a]	14/33[a]	9/16[a]	5/17[a]
	%	26	35	17	42	56	29

Within a questionnaire category, different letters for responses within a column indicate significant differences (test of proportions $P<0.05$)

Table 3. Symptom Questionnaire Response

Part 1: Percentage of Subjects Reporting Specific Symptoms Immediately Post-Exposure and at 24 Hours

Exposure		Symptom					
		Cough	Phlegm	CT	Wheeze	SOB	DB
California	Immediate	25%	14%	3%	0%	3%	11%
	24-hr Post-Exposure	32%	24%	15%	3%	9%	9%
Tx Unwashed	Immediate	53%	14%	22%	11%	8%	11%
	24-hr Post-Exposure	32%	25%	22%	6%	6%	9%
Tx Washed (1)	Immediate	34%	6%	11.4%	3%	9%	9%
	24-hr Post-Exposure	38%	25%	31%	9%	9%	9%
Rayon	Immediate	14%	11%	9%	3%	11%	6%
	24-hr Post-Exposure	24%	33%	12%	6%	9%	9%
Tx Washed (0.5)	Immediate	25%	17%	17%	0%	4%	4%
	24-hr Post-Exposure	33%	20%	27%	0%	7%	7%

Part 2: Number of Atopic and Non-Atopic Subjects Reporting Specific Symptoms (# with symptoms/total number)

		A	NA	A	NA	A	NA	A	NA	A	NA	A	NA
California	Immediate	5/17	4/19	3	2	1	1	0	0	1	0	3	1
	24-hr Post-Exposure	7/17	4/16	6	2	4	1	1	0	3	0	3	0
Tx Unwashed	Immediate	9/16	10/20	4	1	7	1	3	1	3	0	4	0
	24-hr Post-Exposure	7/15	2/17	6	2	5	2	1	1	2	0	3	0
Tx Washed (1)	Immediate	6/17	6/18	2	0	2	2	0	1	3	0	2	0
	24-hr Post-Exposure	7/16	5/16	6	2	7	2	2	1	2	1	2	1
Rayon	Immediate	2/17	3/18	2	2	2	1	1	0	4	0	2	0
	24-hr Post-Exposure	5/16	3/17	7	4	3	1	2	0	3	0	3	0

Table 4. Mean Airborne Dust Levels, Total Bacteria, and Endotoxin for Each Exposure Condition

	Dust Level[1]	Log Total Bacteria
Exposure	mg/m^3 (sem)	CFU/m^3 (sem)
Rayon	0.46 (0.02)	2.93 (0.013)[a]
California	1.05 (0.03)	3.15 (0.086)[a]
Texas Unwashed	1.02 (0.02)	5.07 (0.084)[b]
Texas Washed (1.0)	1.00 (0.02)	4.52 (0.045)[c]
Texas Washed Week Five (0.5)	0.49 (0.02)	4.14 (0.044)[d]
Clean Room	ND	3.16

[1]Mean of four exposure days
Means with different letters indicate significant differences;
Duncan's multiple comparison (P= 0.05)

Table 5. Mean Endotoxin Levels for Different Exposures

Exposure	Lint (Eu/mg)	Airborne (Eu/mg)	Dust (Eu/m^3)
Rayon	2.27	454.9 (86.7)[a]	209
California	81.93	775.5 (113.5)[a]	814
Texas Unwashed	259.62	5735 (824)[b]	5850
Texas Washed (1.0)	72.37	478.3 (54.4)[a]	478

Means with different letters indicate significant differences;
Duncan's multiple comparison (P= 0.05)

Table 6. Pre-test FEV_1 and FVC Over the Four Weeks

		Atopic					Non-Atopic			
		FEV_1		FVC			FEV_1		FVC	
Week	n	Means	SD	Means	SD	n	Means	SD	Means	SD
1	15	104.2	(13.3)	105.0	(16.1)	22	101.2	(10.1)	99.5	(10.5)
2	14	102.5	(12.1)	101.7	(13.7)	23	97.6	(12.5)	96.5	(10.8)
3	15	103.3	(12.8)	103.3	(14.7)	20	99.1	(13.4)	97.6	(11.0)
4	15	102.4	(11.3)	102.5	(13.8)	19	98.2	(11.6)	95.2	(10.3)

Table 7. General Linear Model Comparing Pre-Exposure Ventilatory Response Over the Four Weeks of the Study[a.b.c]

	FEV_1			FVC		
Week	LS Mean	SEM	≠0	LS Mean	SEM	≠0
1	102.6^{A}	(0.52)	Y	102.4^{A}	(0.51)	Y
2	100.4^{B}	(0.52)	Y	99.7^{B}	(0.52)	Y
3	100.6^{B}	(0.54)	Y	99.9^{B}	(0.53)	Y
4	99.5^{B}	(0.54)	Y	98.6^{B}	(0.54)	Y

[a]FEV_1 Model which includes atopic status indicates an effect of week ($p < 0.0007$)
Model which includes atopic status indicates an effect of exposure ($p < 0.05$)
No effect of atopy ($p = 0.2$)
[b]FVC Only an effect of week ($p < 0.0001$)
Exposure ($p = 0.086$)
Atopy ($p > 0.1$)
[c] Ratio No effect of week, exposure or atopy.
Different letters within a column indicate means are significantly different ($P < 0.05$).

Table 8. Pre-exposure Ventilatory Response of Non-Atopics[a]

	FEV_1			FVC		
Week	LS Mean	SEM	≠0	LS Mean	SEM	≠0
1	100.7^{A}	(0.64)	Y	99.7^{A}	(0.80)	Y
2	97.6^{B}	(0.62)	Y	$96.4^{B,C}$	(0.58)	Y
3	98.0^{B}	(0.68)	Y	96.7^{C}	(0.63)	Y
4	96.8^{B}	(0.70)	Y	94.8^{B}	(0.66)	Y

[a]Significant effect of week ($p < 0.0008$) and exposure ($p < 0.04$)
Different letters, within a column, indicate means are significantly different ($P < 0.05$).

Table 9. Pre-exposure Ventilatory Response of Atopics[a]

	FEV_1			FVC		
Week	LS Mean	SEM	≠0	LS Mean	SEM	≠0
1	103.8	(0.89)	Y	104.7	(0.96)	Y
2	103.8	(0.94)	Y	103.2	(1.02)	Y
3	103.0	(0.89)	Y	103.1	(0.96)	Y
4	102.7	(0.89)	Y	102.7	(0.96)	Y

[a]No effect of week or exposure ($p > 0.7$)

Table 10. Pre-Exposure Methacholine Responsiveness[a]

	Atopic				Non-Atopic			
Week	n	Mean	SD	Range	n	Mean	SD	Range
1	15	-1.22	(1.75)	(-0.16, -7.0)	22	-0.42	(0.58)	(0.064, -2.52)
2	14	-1.52	(2.57)	(-0.13, -10)	23	-0.38	(0.33)	(0.162, -1.15)
3	15	-1.29	(1.93)	(-0.01, -7.9)	21	-0.59	(0.29)	(-0.06, -4.23)
4	15	-1.47	(1.77)	(-0.01, -6.7)	18	-0.38	(0.29)	(-0.007, -1.09)

[a]Analysis Excluding Subject #33; data reported as the slope of the line of the saline value FEV_1 and log cumulative dose of methacholine (the larger the negative number the greater responsiveness).

Table 11. Pre-Exposure Methacholine Slope[a]

Week	LS Mean	SEM	≠0
1	-0.84	(0.09)	Y
2	-0.91	(0.09)	Y
3	-0.95	(0.09)	Y
4	-0.96	(0.09)	Y

[a]Analysis shows no effect of week but Atopy a significant term ($p < 0.05$). Exposure is not significant ($p > 0.2$).

Table 12. Summary Data for Pre-Exposure Methacholine Responsiveness for Atopics and Non-Atopics Pooled over the Four Week Study[a]

Atopy	LS Mean	SEM	≠0
No	-0.47[A]	(0.28)	N
Yes	-1.36[B]	(0.33)	Y

[a](A≠B; $p < 0.05$)

Table 13. Comparison of Pre-Exposure Methacholine Slopes for Atopics and Non-Atopics[a]

	Atopic			Non-Atopic		
Week	LS Mean	SEM	≠0	LS Mean	SEM	≠0
1	-1.25[B]	(0.14)	Y	-0.42[A]	(0.12)	Y
2	-1.47[B]	(0.15)	Y	-0.39[A]	(0.11)	Y
3	-1.30[B]	(0.14)	Y	-0.57[A]	(0.12)	Y
4	-1.44[B]	(0.14)	Y	-0.48[A]	(0.13)	Y

[a](A ≠ B; $p < 0.001$)

Table 14. Pre to Post-Exposure Change in FEV_1[a] (Pre-Post Liter), FVC[a], and Ratio[b] (Pre-Post Change in Ratio)

		Non-Atopic			Atopic		
Parameter	Exposure	n	$\bar{x}$	SD	n	$\bar{x}$	SD
FEV_1	California	21	0.009	(0.12)	15	0.073	(0.19)
	Tx Unwashed	21	0.106	(0.16)	15	0.123	(0.13)
	Tx Washed (1)	20	0.026	(0.15)	15	0.083	(0.13)
	Rayon	22	0.014	(0.07)	14	0.033	(0.13)
	Tx Washed (0.5)	20	0.020	(0.21)	13	0.046	(0.13)
FVC	California	21	0.013	(0.12)	15	0.059	(0.13)
	Tx Unwashed	21	0.121	(0.16)	15	0.183	(0.17)
	Tx Washed (1)	20	0.057	(0.16)	15	0.099	(0.12)
	Rayon	22	0.050	(0.12)	14	0.017	(0.12)
	Tx Washed (0.5)	20	0.021	(0.09)	12	0.037	(0.09)
Ratio	California		0.003	(0.02)		0.0004	(0.02)
	Tx Unwashed		0.011	(0.02)		0.022	(0.03)
	Tx Washed (1)		0.009	(0.03)		0.009	(0.02)
	Rayon		0.009	(0.03)		-0.001	(0.02)
	Tx Washed (0.5)		0.007	(0.07)		-0.002	(0.02)

[a]Liters (positive values indicate a reduction in function during exposure)
[b]Change in Ratio

Table 15. Model Evaluation of Pre to Post-Exposure Spirometry[a,b]

	FEV_1		FVC		Ratio	
Exposure	LS Mean	≠0	LS Mean	≠0	LS Mean	≠0
California	0.040[A]	Y	0.041[A]	N	0.003[A]	N
Tx Unwashed	0.149[B]	Y	0.117[B]	Y	0.015[B]	Y
Tx Washed (1)	0.072[A]	Y	0.056[A]	Y	0.007[A]	Y
Rayon	0.042[A]	Y	0.028[A]	N	0.005[A]	N

[a]Controlling for Atopic Status there is an effect of exposure. On FEV_1, FVC and Ratio, FEV_1 exposure $p < 0.0001$; FVC exposure $p < 0.03$; and ratio exposure $p < 0.05$
[b]Letters within a column indicates means are significantly different.
Different letters, within a column, indicate means are significantly different ($P < 0.05$).

Table 16. Summary Data for Spirometry Response for Atopics and Non-Atopics Pooled over the Four Week Study (Overall Response of All Individuals to All Cottons)

	FEV_1		FVC		Ratio	
Atopic	LS Mean (SEM)	≠0	LS Mean (SEM)	≠0	LS Mean (SEM)	≠0
Yes	0.08[A] (0.026)	Y	0.078[A] (0.020)	Y	0.007[A] (0.004)	N
No	0.062[A] (0.022)	Y	0.044[A] (0.017)	Y	0.007[A] (0.004)	N

Different letters, within a column, indicate means are significantly different ($P < 0.05$).

Table 17. Pre to Post-Exposure Spirometry Response Excluding Unwashed Cotton[a,b]

	FEV_1			FVC			Ratio		
Exposure	LS Mean	SE	≠0	LS Mean	SE	≠0	LS Mean	SE	≠0
California	0.042	0.02	Y	0.042	(0.02)	N	0.003	(0.003)	N
Tx Washed (1)	0.070	0.02	Y	0.055	(0.02)	Y	0.007	(0.003)	Y
Rayon	0.041	0.02	Y	0.029	(0.02)	N	0.004	(0.003)	N

[a]Exposures are not significantly different
FEV_1 ($p = 0.4$)
FVC ($p = 0.7$)
Ratio ($p = 0.6$)
[b]Atopy not significant; $p > 0.1$ for all variables

Table 18. Pre to Post-Exposure Response of Atopics and Non-Atopics to Rayon, California, and Washed Cotton[a]

		Atopic			Non-Atopic		
Parameter	Exposure	LS Mean	SEM	≠0	LS Mean	SEM	≠0
FEV_1	California	0.059	(0.03)	Y	0.02	(0.02)	N
	Tx Washed (1)	0.099	(0.03)	Y	0.047	(0.03)	N
	Rayon	0.015	(0.03)	N	0.054	(0.02)	Y
FVC	California	0.073	(0.003)	Y	0.014	(0.03)	N
	Tx Washed (1)	0.083	(0.003)	Y	0.028	(0.03)	N
	Rayon	0.032	(0.004)	N	0.019	(0.03)	N
Ratio	California	0.0004	(0.004)	N	0.005	(0.004)	N
	Tx Washed (1)	0.009	(0.005)	N	0.006	(0.004)	N
	Rayon	-0.001	(0.005)	N	0.009	(0.004)	Y

Table 19. Ventilatory Response of Atopics to Rayon, Texas Washed, and California Cotton [a]

	FEV_1			FVC			Ratio		
Exposure	LS Mean	SEM	≠0	LS Mean	SEM	≠0	LS Mean	SEM	≠0
California	0.017	(0.02)	N	0.006	(0.02)	N	0.004	(0.004)	N
Tx Washed (1)	0.041	(0.02)	N	0.024	(0.03)	N	0.005	(0.005)	N
Rayon	0.061	(0.02)	Y	0.024	(0.02)	N	0.01	(0.004)	Y

[a] For Non-Atopic (excluding Atopics from the analysis), no effect of exposure

FEV_1 $p = 0.3$

FVC $p = 0.8$

Ratio $p = 0.6$

Table 20. Response of Non-Atopics to Rayon, Texas Washed, and California Cotton

	FEV_1			FVC			Ratio		
Exposure	LS Mean	SEM	≠0	LS Mean	SEM	≠0	LS Mean	SEM	≠0
California	0.077	(0.03)	Y	0.085	(0.05)	N	0.003	(0.005)	N
Tx Washed (1)	0.010	(0.03)	Y	0.080	(0.05)	N	0.011	(0.005)	Y
Rayon	0.002	(0.03)	N	0.026	(0.05)	N	-0.004	(0.005)	N

[a] For Atopic (excluding Non-Atopics), NO effect of exposure
FEV_1 $p > 0.1$
FVC $p > 0.6$
Ratio $p > 0.7$

Table 21. Change in FEV_1 - Methacholine Dose Slope* After Exposure

Exposure	Atopics	Non-atopics
Texas Cotton (TC)	2.79	2.08
Washed Cotton (WC)	2.53	1.41
California Cotton (CC)	1.54**	-0.61
Rayon (R)	0.41	0.28

*Positive value indicates increase in responsiveness
**P=0.4 for difference from non-atopics

1992 Study

Introduction

A recent study has demonstrated that textile workers who smoke may be at increased risk for chronic impairment from exposure to cotton dust (*51*). Pulmonary function tests of a study population of 2,659 textile workers were evaluated in a 5 time and multiple regression used to relate the resulting slopes to indices of exposure while controlling for age, gender, individual mill, smoking category, and percentage of cotton in the cotton blend. A total of nine textile mills (6 cotton/3 synthetic) were included in the study. Using the annual change in FEV_1 predicted from the study for 40 year old male (yom) workers, the study predicted that at 150 ug m^{-3}, 50 ug below the current dust standard, smoking textile workers would have an average annual decline of 41.2 ml yr^{-1}. This compares to a decline of 40 ml yr^{-1} for 40 year-old-male controls. At 200 ug m^{-3}, the current cotton dust standard, an annual decline of 49.3 ml yr^{-1} was predicted and -57.3 ml was predicted at 250 mg m^{-3}. A similar trend was observed for women. These data indicate that workers that smoke may be at increased risk for abnormal loss of lung function in cotton textile environments in compliance with the current cotton dust standard. The contribution of atopy to the accelerated loss of function in cigarette smokers was not evaluated in the study. In a study of naive volunteers (*52*) no difference was observed in the acute pulmonary response of smokers and non-smokers; however, a significant interaction was observed between increasing age and increase acute responsiveness among smokers. These data suggest that chronic cigarette smoking is a risk factor for development of an acute ventilatory response.

Previous studies have demonstrated that atopy, as determined by questionnaire or an objective measure such as skin test or total IgE, is a risk factor for both acute declines in FEV_1 and increased airway reactivity in both naive subjects and cotton textile workers exposed to cotton dust (*53-55*). Specific experimental studies designed to evaluate the response of subjects that smoke to aerosols of cotton dust have not been done. Most studies have attempted to eliminate smoking as a variable. Considering the previously cited studies and that in the United States smokers comprise 30-40% of the cotton textile work force specific studies evaluating this group are needed. Furthermore, based on our studies in 1990 and 1991, demonstrating that atopics are more responsive than non-atopics, smokers should be evaluated after stratification by atopic status.

The following objectives were specifically proposed for study in 1992:

1. Are there differences in the acute overshift response in ventilatory parameters or non-specific bronchial reactivity between naive subjects who smoke and naive subjects that do not smoke when exposed to aerosols of cotton dust.

2. Is the acute response, as measured by FEV_1, a valid predictor for chronic impairment in textile workers that smoke? (also see specific objective 1 for 1991)

3. Do cottons with variable levels of endotoxins cause a dose dependent response in either non-specific bronchial responsiveness or acute change in FEV_1 in naive subjects who

smoke?

4. Do specific treatments of cotton, such as washing, reduce the response to cotton dust measured by other markers such as bronchial responsiveness in naive subjects that smoke?

5. Is the response to aerosols of cotton dust containing variable levels of endotoxin (or other constituents) different in atopics that smoke than that of smoking non-atopics?

6. Does the addition of an oil overspray alter the ventilatory response or bronchial reactivity to aerosols of cotton dust or alter the physical or biological characteristics of the dust?

Methods

Subject Recruitment and Selection: Smokers without a history of asthma or previous exposure to cotton dust were recruited by radio and newspaper advertisement. Subjects were initially screened by telephone and if qualified, invited to a follow-up screening session at the laboratory. Laboratory screening consisted of spirometry, administration of a more detailed medical and occupational questionnaire, and if not excluded by questionnaire response, evaluation of atopic status by skin test. The questionnaire asked about personal and family history of allergy and previous exposure in dusty environments. Women of child bearing age were asked to complete a pregnancy test. Criteria for exclusion from the study included an FEV_1 or FVC <80% predicted; FEV_1/FVC ratio <70%; positive pregnancy test; previous history of exposure to cotton dust; or chronic respiratory illness. Pack-years smoked was determined by each subject estimating the averaged number of cigarettes smoked per day and the number of years smoked.

Skin testing was performed using a sterile, disposable multiple head applicator (Multi-test^{tm}; Lincoln Diagnostics, Inc) with a battery of six inhalant allergens common to the southeastern Unites States. The allergens included: cat dander, ragweed, a southern-grass mix, pecan-hickory tree mix, mite mix, and Alternaria sp. (Greer Inc.). Histamine (1:1000 wt/vol) and saline with 50% glycerine were used as a positive and negative control respectively. The wheal associated with the histamine and saline responses were evaluated at 10 minutes after application and at 15 minutes for the specific allergens. Erythema was not used in the evaluation of atopic status. For this study, subjects were categorized as atopic if they has a positive response relative to the negative control to one or more of the specific allergens.

Experimental Design and Exposure Sequence: Subjects selected for the study were asked to participate two consecutive days a week for four weeks. Each week participants reported the day prior to dust exposure for methacholine bronchoprovocation testing. The next day, prior to entering the cardroom, participants completed baseline spirometry. Each participant remained in the cardroom for 5 hours during which lunch was provided; restroom breaks were allowed as needed. Subjects were removed from the cardroom after 5 hours and spirometry and methacholine testing performed. A symptom questionnaire was completed immediately post exposure and again at 24 hours. The same pre test and exposure day was maintained for each subject during the four week study.

Measurements of lung function and airway reactivity: Ventilatory function was tested with standard spirometry meeting American Thoracic Society guidelines (10). A Ohio Medical Products 822 rolling seal spirometer was used for all spirometric evaluations. Ventilatory function was compared to predicted values (11) and only those subjects whose FEV_1 was equal to or above 80% of the predicted value were retained for further evaluation for the study. Each subject performed a graded dose methacholine bronchoprovocation test on the day prior to each exposure. Methacholine solutions were prepared in concentrations of 1.25, 5, and 20 mg/ml in normal saline using Provocholine® (Roche Laboratories, Nutley, NJ). Administration of solutions consisted of 5 breaths from a DeVilbis 626 nebulizer triggered by a Rosenthal-French dosimeter (Laboratory for Applied Immunology, Baltimore, MD) for 0.6 seconds at 20 psi. The subjects began each inhalation from functional residual capacity, inhaled to total lung capacity, and held the inspiration for three seconds. Five inhalations were taken sequentially and timing started. At three minutes after the last inhalation a maximal forced expiratory maneuver was performed to measure FVC and FEV_1. Unless a technical defect in the first maneuver occurred (e.g., a cough within the first second of expiration) the FEV_1 from a single maneuver was used as the value recorded for analysis. The next series of inhalations was begun 5 minutes after the completion of the previous series. During the three minutes between completing the inhalations and measurement of the FEV_1, the subjects were coached to breath normally and not to take deep breaths or purposely cough. The FEV_1 after each dose was compared to that after 5 breaths of saline at the start of the challenge test and the next higher dose of methacholine given unless the FEV_1 had fallen by 20% or more from the post-saline value. Each solution was inhaled from a set of labeled nebulizers assigned to the subject for the entire study so that the same nebulizer was used for a given solution each time the subject was tested. The nebulizers were emptied after each session, cleaned and dried, and fresh solutions added just before the next test.

Results from methacholine challenge were evaluated in two ways. For the first method, the percent change in FEV_1 from the post-saline value was calculated at the highest dose of methacholine common to both pre- and post exposure for each subject. For week to week pre-exposure responsiveness, the HCD common to all four weeks was used for comparison. For the second method, the dose response slope was calculated for each challenge using least squares linear regression of the percent change in FEV_1 from the post-saline value against the log of the cumulative inhaled dose of methacholine. The fitted line was forced to cross the point of no fall in FEV_1 at zero dose of methacholine.

Study Cottons: Four exposure conditions were evaluated during the first four weeks of the study. Dust was generated from four types of fiber: Rayon, Texas unwashed cotton, Texas unwashed cotton with an oil overspray to suppress dust, and the Texas cotton after washing. A Texas grown cotton, predominantly 1990 crop, grade code 41 (USDA) was blended to make a 34 bale mix. For the exposures, subsets of this cotton were either washed, treated with a dust suppressant overspray prior to the exposure, or left untreated. The cotton was washed at 93C using a commercially available batch washing process. A commercially available overspray Milube N-32 was applied at a rate of 0.15%, a application rate considered low for normal processes, at the opening line prior to exposure. The rayon was a commercially available standard textile grade fiber characterized as 1.5 denier, 1.5 inches, and bright. The target dust

level for each cotton was 1 mg/m^3. The target dust level for rayon was 0.5 mg/m^3 which was the maximum achievable for the carding conditions and minimum room ventilation criteria.

Exposure Room and Environmental Monitoring: All exposures took place in an experimental cardroom at the USDA-ARS Cotton Quality Research lab in Clemson, South Carolina. The dimensions of the cardroom have been described previously (8). Chairs for subjects were situated around the perimeter of the cardroom and subjects were asked to move to a different location at hourly intervals. Dust levels were controlled by adjustment and balance of card production rates and air supply and exhaust rates (9). Dust levels were monitored using four vertical elutriators (VE) at different positions in the cardroom and with a portable continuous aerosol monitor (PPM Inc, Knoxville, TN). The particle size distribution was monitored using an Anderson Series 210 Cascade Impactor. All cottons and rayon were processed through blender-feeders and three stages of blending, opening, and cleaning on a finisher picker to prepare laps for feeding a carding machine. Carding was at 25-30 lbs/hr for the cotton and 60 lbs/hr for the rayon.

Ambient air was sampled for total viable airborne bacteria with six-stage Anderson air samplers (Anderson 2000 Inc., Atlanta, Georgia) operated at 0.028 m^3/min for 15, 30, 60, or 120 seconds. The samplers were loaded with 90 mm plastic petri plates filled with 40 ml of trypticase soy agar [30 g trypticase soy broth, 20 g agar and 5 g yeast extract per liter and cycloheximide (50 μg/ml)]. The plates were incubated for 22-30 hours at room temperature (RT;24.1±03°C).

For airborne endotoxin, VE filters were extracted in 50 ml pyrogen free water (PFW) for 1 hour in pyrogen free borosilicate glass containers. Appropriate serial 10-fold dilution were evaluated by the kinetic chromogenic modification of the Limulus amebocyte lysate (LAL) assay (BioWhittaker).

Data Analysis: To adjust for differences in age, height, and sex, baseline spirometric data were converted to percent predicted using Knudson and co-workers equations (10) adjusted for race. All four groups were combined to determine the response to each type of exposure. Data were analyzed using the General Linear Model procedure for analysis of variance and least squares adjustment of means for missing data (SAS, Cary, NC). Differences were considered significant at the $P \leq 0.05$ level. For viable microbial counts the data were converted to logs and evaluated by GLM ANOVA. Multiple comparison was done using Duncan's range test.

Results

Demographic Data: A total of 34 subjects participated in the study. Demographic data are given in Table 1. Eighteen subjects were identified as atopic based on positive skin test. There were no significant difference in age, sex, baseline spirometry, or pack-years smoked for atopic and non-atopic subjects.

Skin Testing: For the 18 subjects with a positive skin test, nine were positive to a single antigen and nine were positive to two or more antigens. No subject responded to more than 4 antigens. Overall, there were 15 responses to mite antigen, 6 to the grass mix, 5 to the ragweed, 4 to cat,

and 2 to the tree mix.

In a follow-up evaluation, the response to the following additional antigens was evaluated: fescue, Bermuda grass, dog, penicillin, aspergillis, dog dander, cockroach, mixed feathers, and cotton linters. Based on the response to these antigens one additional subject was identified as atopic based on using the criteria of a single positive skin test to determine atopic status; however since that subject was specified as a non-atopic in the initial screening no change was made in the analysis of the methacholine or spirometric data. One subject, previously designated as atopic, gave a positive skin response to cotton linters.

Follow-up testing was also done with the same antigens that were used for screening. Four subjects that were positive to a single antigen during screening were negative on the second test. Evaluation of the four subjects (Subjects # 4, 32,42,44) as a subgroup demonstrated no difference in either demographic or response parameters when visually compared to the overall analysis of the atopics and nonatopics (data not shown). However, these data would suggest that atopic status assigned as a single positive skin response may not be valid and that a minimum of two or more positive responses would be a better indicator.

To determine if there were trends related to the number of positive skin test, the data were sorted by number of positive skin test (0, 1, or >1) and baseline and response variables visually compared (Tables 2 and 15). The baseline data are shown in Table 2. Nine subjects had more than one positive skin test, nine were positive to a single antigen, and 16 were skin test negative. Subjects with more than one positive skin test tended to have smoked fewer pack-years, be younger, have lower percent predicted FEV_1 and FVC, and have a higher baseline methacholine responsiveness. On average, the baseline methacholine responsiveness, as determined by slope, appears to be less in smokers defined as atopic than in atopic non-smokers in the prior study.

Table 12 from 1991 reports that baseline methacholine responsiveness in non-smoking non-atopics was -0.47 (0.28 SEM) and -1.36 (0.33 SEM) for non-smoking atopics. For subjects in this study with two or more positive skin tests, baseline responsiveness was -0.73 (0.55 SD) and for smoking non-atopics -0.59 (0.7 SD). These data suggest that the atopic smokers tended to have a reduced baseline non-specific reactivity relative to the atopic non-smokers. Reactivity in non-atopic smokers and non-smokers appears to be similar. It should be noted that the method to determine atopic status differed between years (i.e. serum test for IgE in 1991 and skin test in 1992). The differences in reactivity found may reflect: 1) a tendency of the more reactive atopics to refrain from smoking due to symptoms; 2) a difference in the categorization of atopic status by the two methods; or 3) a chance finding. Although the data were not analyzed statistically, the differences are small and do not appear to be clinically significant.

Baseline and response data were also sorted by pack-years smoked (≤ 10, 11-20, or >20 years) (Tables 3 and 14). Subjects who had smoked the longest (>20 pack-years) were older than the subjects in the other smoking categories and had lower ventilatory function as indicated from the percent predicted for FEV_1 and FVC. The relative number of atopics was similar among groups and there appeared to be no major differences in baseline methacholine responsiveness with number of pack-years smoked. The data were also sorted based on 10 or less pack years smoked

or greater than 10 pack years smoked. The trends were similar to those observed in Table 3 (data not shown).

There was no association between baseline methacholine responsiveness (methacholine slope at screening) and subject age, pack-years smoked, or number of positive skin test. These data are in agreement with previous studies reporting that smokers are not more likely than non smokers to display more positive allergy skin test (Conner). There was a non-significant association of lower baseline FEV_1 and increased airway responsiveness (Figure 1). The trend demonstrates that subjects with a baseline FEV_1 of <100% predicted have greater methacholine responsiveness.

Screening Questionnaire Response: Subjects were evaluated for atopic status by questionnaire. Only 4 of 34 subjects indicated they were atopic by questionnaire (3 physician confirmed and 1 non-physician confirmed). There was no agreement between the questionnaire determined response and atopy as determined by skin testing ($p>0.6$; Fishers's exact test 2-tailed).

As was done in 1990 and 1991, the positive predictive value for the questionnaire for determining true atopic status (one or more positive skin tests) was determined. The PPV was 100% in that all three of the subjects indicating physician confirmed atopy by questionnaire were atopic by skin test (Table 4). However, the negative predictive value (NPV) was 52%, indicating that almost 50% of those who indicated they were not atopic by questionnaire had one or more positive skin tests. These data agree with the results from the study in 1991 that the question (physician confirmed hayfever) is not a predictive indicator of atopic status.

There was no significant relationship between atopic status as determined by skin testing and any of the other questionnaire variables evaluating either a personal history of atopy (eczema, food, metals, dust, or animals) or a family history to the same categories of substances ($p>0.6$; Fishers's exact test 2-tailed). The summary screening questionnaire responses are shown in Table 5.

Baseline Pre-exposure data: Baseline spirometry was evaluated prior to each exposure (Table 6). For both FEV_1 and FVC, there was a significant difference between weeks in the group mean pre-exposure spirometry data ($p<0.001$). For both FEV_1 and FVC the percent of predicted was significantly higher for week one; there was a decreasing trend over the remaining three weeks of the study. While not evaluated statistically, the baseline value for the first week did not appear to differ from the values from subject screening (Table 1). There were no significant differences in the pre-exposure FEV_1/FVC ratio over the four weeks.

Exposure was included in the model to determine if the difference in pre-exposure ventilatory response was related to an exposure carryover effect, and was shown not to be significant ($P > 0.6$). These data would suggest that the decrease over the four week period was a negative learning effect, with decreasing effort without change in FEV_1/FVC ratio.

Although atopic status was not a significant predictor variable in the model ($P > 0.3$), previous studies had shown that subjects categorized as atopic had different ventilatory and methacholine

responsiveness baselines. The data in Table 7 show that the trend in decreasing baseline pre-exposure FEV_1 and FVC over the four week study was consistent for both atopics and non-atopics.

Baseline Methacholine Responsiveness: A significant effect of week was also observed in the pre-exposure methacholine response data over the four week study (Table 8, $P<0.001$). For methacholine responsiveness calculated as both the FEV_1 after the highest common dose of methacholine over the four weeks and change in slope, responsiveness in week one was less than the subsequent three weeks. However, only week four was significantly different from week one. There were differences in the trends over the four week study based on the method used to calculate methacholine responsiveness. Using the HCD there was no difference between weeks 1-3, only week four was significantly lower. Using the slope, there was no difference in weeks 1-3, however, weeks two and three were also not different from week four.

There was no difference in the change in methacholine responsiveness over the four week study for atopics and non-atopics ($p=0.47$). There was also no effect of exposure on pre-exposure methacholine responsiveness, suggesting there was not a carryover effect from the previous week's exposure ($p=0.57$). The increase in pre-methacholine responsiveness may have been due to some as yet unidentified variable or a general effect of particulate exposure on the airways which did not differ among the types of exposures. The small drops in pre-exposure FEV_1 would also tend to cause an apparent increase in responsiveness as measured by the FEV_1 after a given dose of methacholine or the slope of the line relating percent fall in FEV_1 against dose.

Exposure Data: Summary environmental data are shown in Table 9. Target dust levels for exposure to Texas washed cotton, Texas unwashed, and Texas overspray were 1 mg/m^3. There was no significant difference in bacterial levels CFU/m^3 for the washed cotton and rayon exposures. Both levels were approximately 10-fold higher than the clean room and 20-fold less than the Texas unwashed cotton.

These data differ from bacterial levels measured in 1991 for similar exposures. In 1991, bacterial levels for Texas washed were approximately 10-fold higher than in 1992 (3.64 vs 4.52 log CFU/m^3) and for rayon was approximately 10-fold lower than in 1992 (3.67 vs 2.93 log CFU/m^3). The levels for the Texas unwashed were similar between the two years (5.02 vs 5.07 log CFU/m^3).

Airborne bacterial levels for the oversprayed cotton were significantly higher than either the rayon or Texas washed, but were significantly lower than the Texas unwashed without overspray. These data would suggest that the overspray either suppresses the release of bacterial laden particles during carding or alters the size distribution of particles, which reduces the viable bacterial counts.

Endotoxin measurements are also shown in Table 9. There was no significant difference in the level of endotoxin between the Texas washed cotton and rayon dust (Eu/mg). Significantly higher levels of endotoxin was found in the Texas unwashed and Texas oversprayed VE dust samples. There was no significant difference in the levels of endotoxin (EU/mg) in the Texas

unwashed and Texas oversprayed When the endotoxin levels were extrapolated to airborne concentrations, the levels for rayon and Texas washed cotton were similar and approximately one log lower than the levels for Texas unwashed and Texas oversprayed. Comparison of endotoxin levels for rayon and washed cotton for 1991 vs 1992 were 454 vs 996 and 478 vs 517 Eu/mg, respectively. The washed cotton was the same for both years, however, in 1992 the rayon was from a different batch and the levels of endotoxin in the dust were higher. This may also account for the differences between 1991 and 1992 for bacteria on the rayon. When these endotoxin data are expressed as Eu/m^3, exposure levels for 1991 vs 1992 for rayon and washed cotton were 209 vs 388 Eu/m^3 and 478 vs 507 Eu/m^3, respectively. There was no difference in the endotoxin exposure levels (Eu/m^3) between washed cotton and rayon for either 1991 or 1992.

Dust Levels: To determine if there were differences in the aerodynamic mass median diameter (AMMD) and geometric standard deviation of the generated dust, a cascade impactor was used to evaluate the dust. Texas unwashed produced the particles with the smallest AMMD and largest geometric standard deviation. Washed cotton had the largest AMMD followed by the Texas unwashed with overspray. The overspray did alter the AMMD from 2.4 μm to 3.1 μm but did not change the standard deviation (Table 10). Comparison to common exposures in 1991 demonstrated that the AMMD in 1992 was in general larger. This may reflect an aging phenomenon of the cotton samples over the year as well as the different batch of rayon. However, too few samples were taken for a complete evaluation of the dust.

Effects of Exposure on Spirometry: Subjects were exposed to aerosols of three different cotton dusts and rayon. The pre to post-exposure changes in FEV_1 are shown in Table 11. Atopic status was shown to be insignificant in the model used to evaluate the ventilatory response to the different exposures ($p= 0.593$). For both washed cotton and rayon there was an increase in FEV_1 over the exposure period (0.012 and 0.027 liters, respectively). The response to washed cotton was not significantly different from the response to rayon, nor were the changes significantly different from 0 (LS mean = 0). The data would indicate that both the washed cotton and the rayon were inert regarding the ventilatory response FEV_1. These data differ from 1991 in which non-smoking subjects responded to rayon and washed cotton with a small decrease in FEV_1 that was significantly different from zero. Although not studied, a sham or no exposure day would be expected to result in a small increase in FEV_1 so this fall would likely have been significantly different from the response to a sham exposure.

Both the unwashed cotton and unwashed cotton with overspray caused decreases in FEV_1 that were significantly greater than either rayon or washed cotton; however, there was not a significant difference between the two unwashed cottons. A similar trend was seen for the FVC and FEV_1/FVC ratio for all four types of exposure (Table 11).

Although atopy was not a significant predictor of responsiveness, previous studies have shown that atopics tend to have greater drops in FEV_1 than non-atopics exposed to cotton dust. The data were evaluated by atopic status to determine if those atopic subjects in this study had a response that is consistent with this observation (Table 12). In general, the atopics tended to be more responsive than non-atopics in both the FEV_1 and FVC response for all exposures except the overspray cotton for which the FEV_1 response was slightly greater for the non-atopics. The

inability to distinguish a significant difference between the response of atopics and non-atopics is likely to result from the small numbers in the study. However, the skin-test response criteria used to categorize the subjects as atopics, or the interaction of smoking status and atopy on ventilatory responsiveness may have contributed to this result.

The data are expressed as percent of predicted in Table 13. The trends are similar to those shown in Tables 11 and 12. For the Texas unwashed and Texas overspray the pre to post-exposure changes in FEV_1 were -4.21% and -3.36% respectively. For the Texas washed and rayon the changes were +0.49% and + 0.86% respectively. For all exposures except the Texas overspray, the atopics were more responsiveness. These data corroborate the findings observed in 1991 for non-smoking atopic and non-atopic subjects. In both years the acute changes in FEV_1 to rayon and washed cotton were not different from each other and both were significantly smaller than those to the unwashed Texas cotton. However, based on these studies, it appears that the smokers have a smaller ventilatory response than non-atopics to the exposures of rayon, Texas washed and Texas unwashed cotton which were common to the studies in 1991 and 1992.

The response parameters of percent change in FEV_1 after exposure and percent change in FEV_1 at the highest common dose of methacholine after exposure for the data sorted by pack-years smoked and number of positive skin test are shown in Tables 14 and 15 respectively. For the data sorted by pack-years smoked (Table 14), there was a small trend of a larger decline in FEV_1 for subjects with greater than 20 pack-years smoked when exposed to Texas unwashed or oversprayed. This trend was not reflected in the change in FEV_1 at the highest common dose of methacholine. The changes for both methacholine and FEV_1 were small for washed cotton and rayon. For the data sorted by number of positive skin test (Table 15), there were no obvious trends in the response parameters based on the number of positive skin tests.

Effects of Exposure on Methacholine Responsiveness: Table 16 shows the data for each exposure using the HCD. There was no significant effect of atopic status on exposure, although there was a consistent trend at the HCD in which atopics had a greater fall post-exposure ($p=0.31$). There was a significant difference between each of the four exposure conditions at the HCD ($p<0001$). For rayon there was a decrease in responsiveness at the highest common dose indicating that the subjects were less responsive after the exposure. These data are in agreement with the lack of responsiveness shown by rayon for FEV_1.

For all cotton exposures there was an increase in responsiveness after exposure. Subjects were most responsive to the Texas unwashed followed by the Texas overspray, Texas washed, and rayon respectively. The significant difference between the rayon and the Texas washed is in agreement with data from 1991 which demonstrated there was not a significant difference between the change in FEV_1 after these exposures, but there was a greater increase in methacholine responsiveness after the exposure to washed cotton.

When the data were evaluated using the slope (Table 17), the washed cotton did produce an increase in reactivity post-exposure, however, it was not significant. It also was not significantly different from the change in responsiveness after the rayon exposure despite the latter showing a slight decrease in responsiveness. Only the Texas unwashed demonstrated an increase in

reactivity that was significantly different from all other exposures. As was observed using the highest common dose, the post-exposure responsiveness to rayon, calculated by the slope, was decreased. For all cottons, post-exposure responsiveness was increased. The change in the slope for both rayon and washed cotton was not significantly from zero, indicating that these dusts were inert. Atopics tended to have larger increases in responsiveness to rayon and the washed cotton and smaller increases to the Texas unwashed and Texas oversprayed cottons than non-atopics, although these differences were not significant. The responsiveness to the Texas overspray cotton was not significantly different from either rayon or Texas washed. However, the change in slope was larger and significantly different from zero. The response was significantly less than the change observed to Texas unwashed. These data would indicate that the overspray does reduce the effect of the dust on bronchial reactivity. However, the dust does remain biologically active.

The question was asked, is the use of common variance by the statistical model valid for exposures that cause a difference in the magnitude of the response. Unwashed cotton was removed from the statistical model and the results of exposure to rayon and Texas washed cotton compared (Table 18). Overall, there was no significant difference in the change in methacholine slope pre to post-exposure between washed cotton and rayon ($p=0.10$) and atopy was a non-significant variable ($p=0.8$). As shown for the complete model, methacholine responsiveness to rayon decreased after exposure and was increased for the washed cotton, although these differences were not significant. Although not significant, atopics had a greater response to the washed cotton than non-atopics, but there was no difference in the response to rayon.

Tables and Figures

Figure 1 Association of Baseline FEV_1 With Pre-Exposure Methacholine Responsiveness

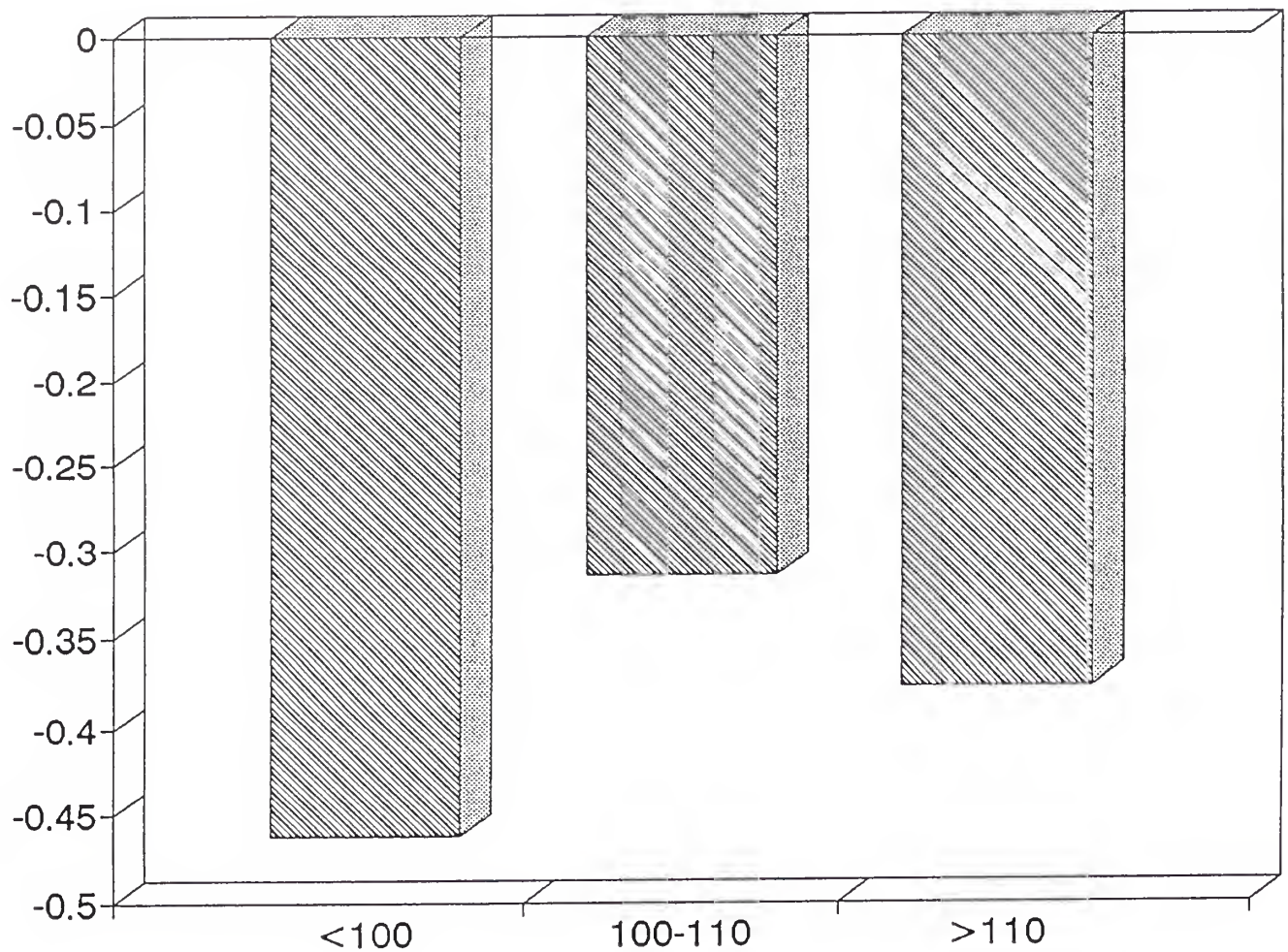

Figure 2 Pre and Post FEV1
Day One and Day Two Exposures

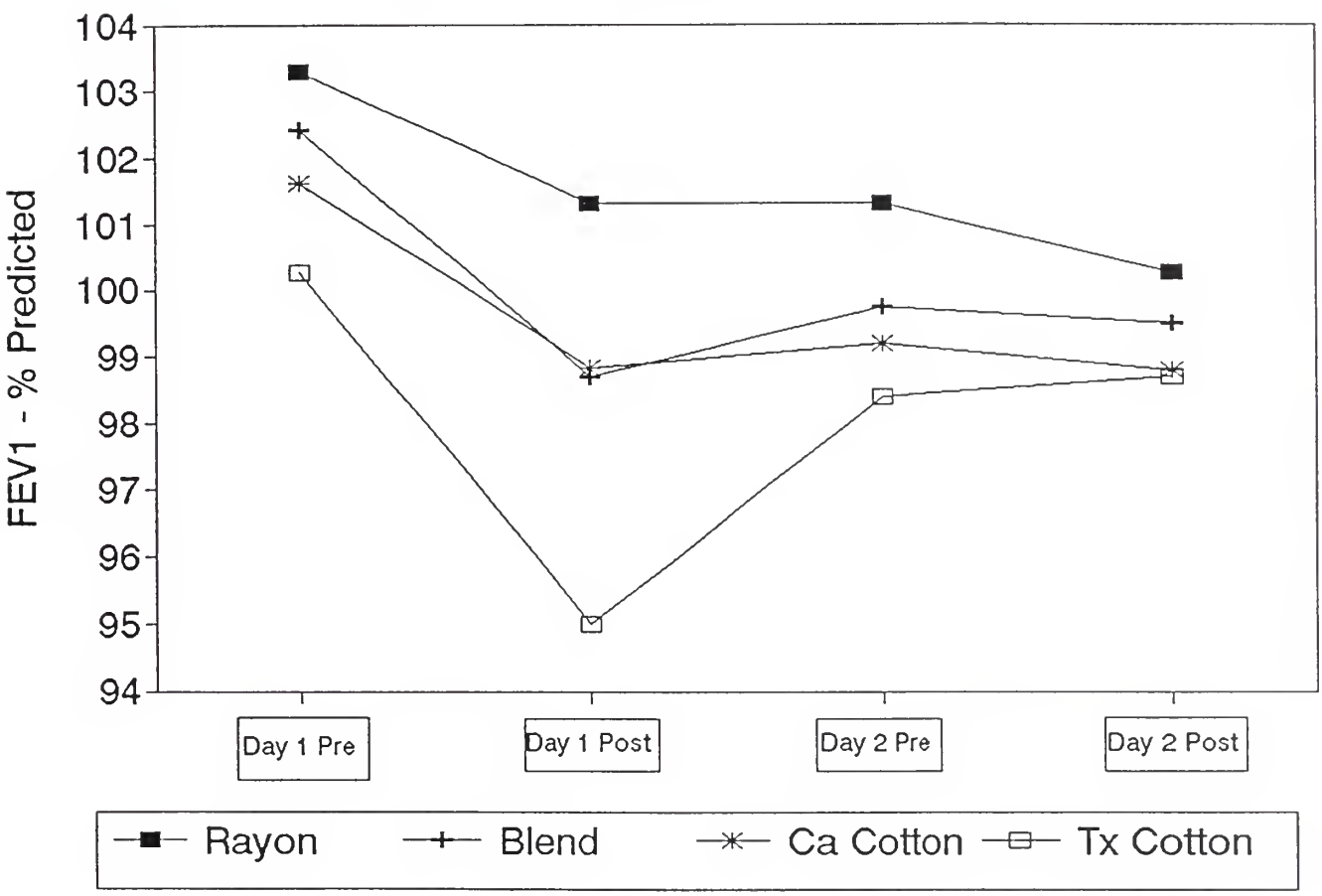

Figure 3 Pre and Post FVC
Day One and Day Two Exposures

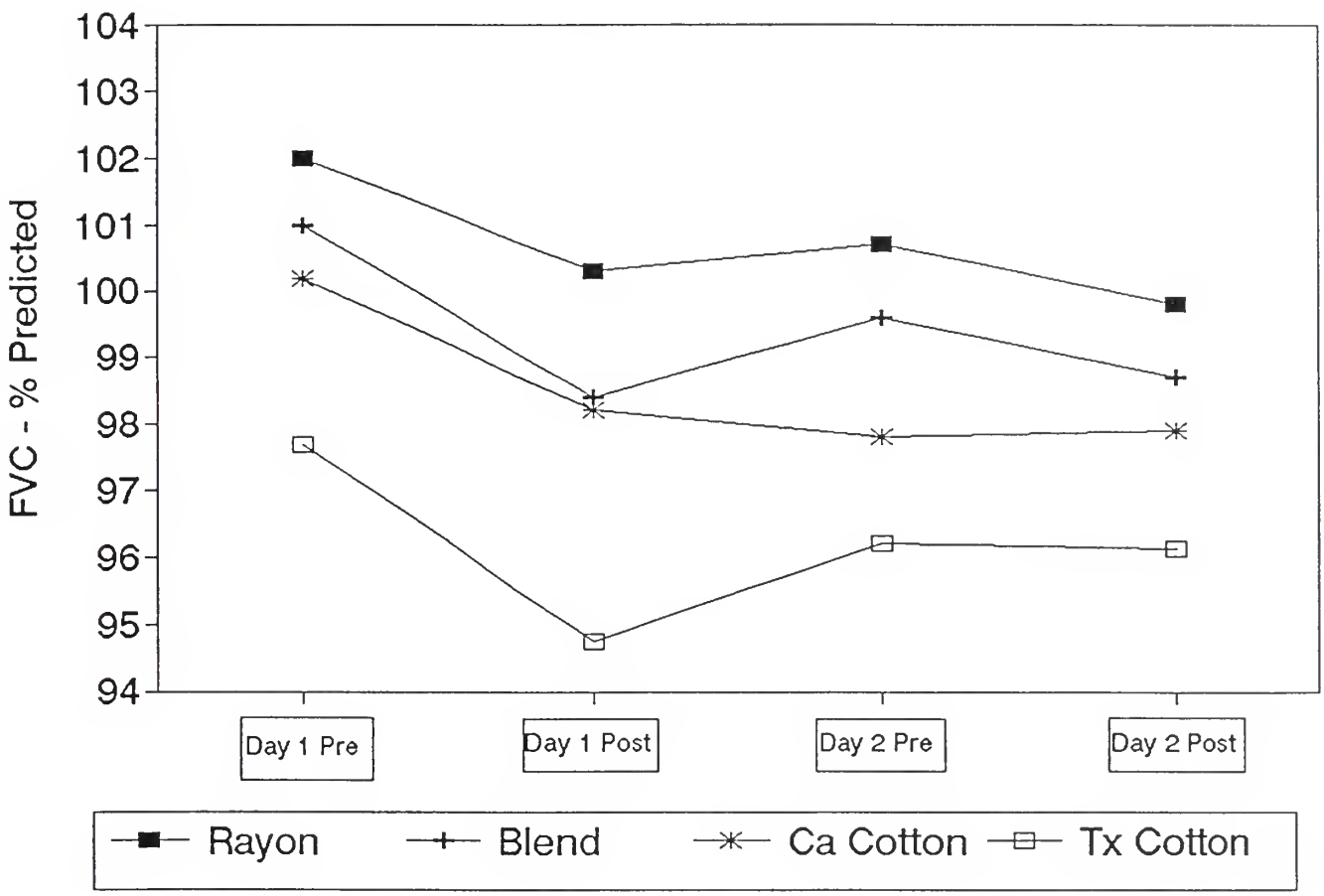

Table 1. Demographic Data

	Total	Atopic	Non-atopic
Subjects	34	18	16
Male	16	10	6
Female	18	8	10
Age	28.8 (8.2)	27.5 (7.3)[a]	30.2 (9.2)
Pack-Yrs	11.6 (10.0)	11.2 (10.4)[a]	12.0 (9.9)
% Pred. FEV_1	104.6 (11.7)	104.6 (13.7)[a]	104.8 (9.4)
% Pred. FVC	105.0 (12.7)	103.2 (14.4)[a]	107.0 (10.6)

[a]Not significantly different from non-atopics. Standard deviation in parentheses

Table 2. Baseline Data Sorted by Number of Positive Skin Test

Positive Skin Test	0	1	>1
Number	16	9	9
Pack Yrs[a]	12.0 (9.5)	12.4 (10)	10.0 (10)
Age[a]	30.2 (8.9)	28.6 (7.9)	26.4 (6.0)
Make:Female	6:10	5:4	5:4
Black:White	3:13	1:8	2:7
% Pred. FEV_1[a]	104.8 (9.1)	106.2 (16.1)	102.9 (9.3)
% Pred. FVC[a]	107.0 (10.2)	105.2 (17.6)	101.2 (8.5)
Ratio (FEV_1/FVC)[a]	0.81 (0.05)	0.84 (0.06)	0.85 (0.05)
Methacholine[a,b]	-0.59 (0.7)	-0.40 (0.3)	-0.73 (0.5)

[a]Mean (SD)
[b]Slope

Table 3. Baseline Parameters for Subjects Sorted by Pack-Years Smoked.

	Smoking Category By Pack Years		
	<10	10-20	>20
n	19	8	7
Positive Skin Test (0/1/>1)	9/4/6	4/3/1	3/2/2
Pack Yrs[a]	4.08 (2.9)	15.21 (2.6)	27.9 (2.7)
Age[a]	24 (5.6)	30.6 (4.7)	39.6 (5.1)
Male:Female	11:8	3:5	2:5
Black:White	4:15	2:6	0:7
% Pred. FEV_1[a]	103.5 (8.0)	113.1 (15.5)	98.1 (5.4)
% Pred. FVC[a]	103.8 (8.1)	114.9 (17.9)	96.8 (5.4)
Ratio (FEV_1/FVC)[a]	0.84 (0.06)	0.809 (0.04)	0.82 (0.04)
Methacholine[a,b]	-0.67 (0.64)	-0.41 (0.2)	-0.54 (0.6)

[a]Mean (SD)
[b]Slope

Table 4. Comparison of Skin-test Determined Atopy to Questionnaire Determined Atopy.

Hayfever By Questionnaire	**Atopic by Skin Test**		
	Yes	No	Overall
Yes	3	0	3
No	15	16	31
Total	18	16	34

Table 5. Summary Questionnaire Response

Response	Personal History						
	HAYF	ECZE	Food	Metal	Dust	Animal	Other
N	30	34	34	33	33	32	29
Y	1			1			3
Y_p	3				1	2	2
	Family History						
N	26	32					30
Y	8	2					4

Y_p: Physician confirmed

Table 6. Baseline Pre-Exposure FEV_1, FVC as Percent of Predicted

Week	FEV_1(SEM)	FVC (SEM)
1	104.3 (0.57)[a]	105.0 (0.48)[a]
2	101.8 (0.62)[b]	102.8 (0.52)[b]
3	101.3 (0.66)[b,c]	102.6 (0.55)[b]
4	99.8 (0.71)[c]	100.9 (0.59)[c]

[1]Significant difference in weeks (P = 0.0001). Means within a column followed by different letters are significant (P < 0.05)

Table 7. Baseline Pre-exposure Ventilatory Response as Percent of Predicted in Subjects by Atopic Status

	FEV_1		FVC	
Week	Non-Atopic (SD)	Atopic (SD)	Non-Atopic (SD)	Atopic (SD)
1	105.0 (11.9)[a]	103.7 (12.5)[a]	103.4 (14.4)[a]	106.7 (13.4)[a]
2	102.5 (12.3)[a,b]	100.4 (12.7)[b]	100.8 (14.7)[a]	103.9 (12.0)[b]
3	102 (12.4)[b,c]	101.0 (12.5)[b]	100 (15.2)[a,b]	103 (12.5)[b]
4	99 (10.9)[c]	98.0 (12.7)[b]	98 (15.7)[b]	98 (10.7)[b]

Means followed by different letters are significantly different (P<0.05). However, atopic status was not significant. Therefore, multiple comparisons should only be used to evaluate trends.

Table 8. Pre-test Methacholine Responsiveness as Measured by FEV_1 at Highest Common Dose or by Slope For Each Week.

Week	HCD[1]	Slope
1	3.156 (0.066)[a]	-0.55 (0.22)[a]
2	3.023 (0.072)[a]	-0.90 (0.24)[a,b]
3	3.025 (0.073)[a]	-0.92 (0.24)[a,b]
4	2.75 (0.0764)[b]	-1.33 (0.25)[b]

Means followed by different letters are significantly different ($P<0.05$).
[1]HCD = FEV_1 after the highest dose of methacholine common to all four weeks on pre-exposure test.

Table 9. Environmental Exposure Data

Exposure	n	VE Dust Level (mg/m^3) (SEM)	Bacterial Level (CFU/m^3) (SEM)	Endotoxin (Eu/mg) (SEM)	Endotoxin (Eu/m^3)
Tx Washed	4	0.98 (0.007)[A]	4,382 (1,053)[A]	517 (51)[A]	507
Tx Unwashed	4	0.98 (0.02)[A]	103,903 (12,956)[B]	4,114 (185)[B]	4,032
Rayon	4	0.39 (0.01)[B]	4,700 (2,582)[A]	996 (57)[A]	388
Tx Overspray	4	1.00 (0.05)[A]	78,288 (8,001)[C]	4,938 (497)[B]	4,938
California			424 (205)		

Means followed by different letters are significantly different ($P<0.05$).

Table 10. Cascade Impactor Data from 1991 and 1992

Cotton	1991		1992	
	AMMD	GSD	AMMD	GSD
Texas Washed	2.4 2.3	2.0 1.9	3.7	2.3
Texas Unwashed	1.4 1.7	2.5 2.7	2.4	2.3
Rayon	2.1	2.1	2.7	2.1
Texas Overspray	NR	NR	3.1	2.3
California	2.1 1.8	2.1 2.4	NR	NR

NR = Not Run

Table 11. Acute Ventilatory Response (Decrease in Liters) to Aerosols of Cotton Dust or Rayon[a]

Exposure	FEV[b] liters (SEM)	LS mean ≠ 0	FVC liters (SEM)	LS mean ≠ 0	Ratio ΔPre-Post (SEM)	LS mean = 0
Tx Washed	-0.012 (0.02)[A]	N	0.052 (0.03)[A]	N	-0.016 (0.008)[A]	N
Tx Unwashed	0.132 (0.02)[B]	Y	0.125 (0.03)[A]	Y	0.009 (0.008)[B]	N
Rayon	-0.027 (0.03)[A]	N	0.023 (0.03)[A]	N	-0.010 (0.009)[A]	N
Tx Additive	0.108 (0.03)[B]	Y	0.087 (0.03)[A]	Y	0.011 (0.008)[b]	N

[a] Data expressed as mean decrease in volume (l), a negative number indicates an increase
[b]Significant effect of exposure ($P < 0.05$)
No affect of atopic status on response ($P > 0.5$).
Means within a column followed by different letters are significantly different ($P < 0.05$).

Table 12. Comparison of the Change in Absolute Values of FEV_1 and FVC (liters) in Response to Aerosols of Cotton Dust or Rayon

	FEV_1 (SEM)		FVC (SEM)	
Exposure	Non-Atopic	Atopic	Non-Atopic	Atopic
Tx Washed	-0.038 (0.04)	0.008 (0.04)	0.034 (0.04)	0.067 (0.04)
Tx Unwashed	0.113 (0.04)[A]	0.146 (0.03)[A]	0.119 (0.04)[A]	0.129 (0.04)[A]
Rayon	-0.052 (0.05)	-0.010 (0.04)	-0.030 (0.05)	0.063 (0.04)
Texas Additive	0.114 (0.04)[A]	0.099 (0.04)[A]	0.076 (0.04)[A]	0.096 (0.04)[A]

[A] Mean significantly different than zero (LS mean = 0; P<0.001)
Values are expressed as absolute volume change (l); negative values represent increase.

Table 13. Acute Percent Changes in FEV_1 in Response to Exposures to Different Cotton Dust and Rayon

	Percent Change in FEV_1					
Exposure	Overall	LS mean ≠ 0	Non-Atopic	LS mean ≠ 0	Atopic	LS mean ≠ 0
Tx Washed	0.49 (0.9)[A]	N	1.52 (1.3)	N	-0.3 (1.2)	N
Tx Unwashed	-4.21 (0.9)[B]	Y	-4.2 (1.4)	Y	-4.3 (1.2)	Y
Rayon	0.86 (1.0)[A]	N	2.3 (1.5)	N	-0.07 (1.2)	N
Tx Additive	-3.36 (0.9)[B]	Y	-4.0 (1.2)	Y	-2.7 (1.2)	Y

Means within a column with different letters are significantly different ($P < 0.05$).
Negative values indicate a decrease from pre-exposure values

Table 14. Response of Subjects to Different Cottons Sorted by Pack-Years Smoked

Exposure		Pack Years		
		≤10	11-20	> 20
Texas Washed[a]	Dust (mg/m^3)	0.98 (0.01)	0.98 (0.01)	0.98 (0.01)
	Bacteria (CFU/m^3)	4,251 (1,777)	4,347 (1,462)	3,939 (1740)
	ΔFEV_1	0.58 (5.3)	-1.81 (4.5)	0.51 (3.01)
	HCD*	-3.8 (10.7)	-6.7 (2.6)	0.46 (8.2)
Texas Unwashed[a]	Dust (mg/m^3)	0.97 (0.03)	0.99 (0.03)	0.97 (0.03)
	Bacteria (CFU/m^3)	103,015 (22,680)	103,056 (25,533)	102,706 (21,918)
	ΔFEV_1	-4.27 (4.3)	-3.72 (3.6)	-6.2 (5.3)
	HCD*	-16.9 (15.5)	-7.0 (11.6)	-19.6 (11.5)
Rayon[a]	Dust (mg/m^3)	0.39 (0.02)	0.39 (0.01)	0.38 (0.02)
	Bacteria (CFU/m^3)	4,293 (4,211)	3,240 (3,476)	3,668 (3,592)
	ΔFEV_1	1.28 (3.5)	-1.05 (4.5)	-0.65 (3.9)
	HCD*	3.04 (14.5)	-2.6 (9.8)	7.6 (11.2)
Texas Overspray[a]	Dust (mg/m^3)	1.00 (0.1)	1.0 (0.11)	1.01 (0.09)
	Bacteria (CFU/m^3)	78,729 (14,107)	88,993 (9,907)	75,191 (13,312)
	ΔFEV_1	-2.8 (4.6)	-2.9 (5.0)	-5.6 (8.2)
	HCD*	-16.6 (13.1)	-2.9 (9.4)	-8.4 (7.4)

[a]Mean (SD)

* HCD = Percent change in FEV_1 from post saline value at the highest dose of methacholine common to all post exposure challenge test.

Table 15. Response of Subjects to Different Cottons Sorted by Number of Positive Skin Test

		Atopy					
		0		1		>1	
Exposure		Mean	SD	Mean	SD	Mean	SD
Texas washed	Dust	0.98	(0.1)	0.98	(0.1)	0.98	(0.01)
	Bacteria	4,326	(1,692)	4,422	(1,816)	3,790	(1,539)
	%ΔFEV$_1$	0.49	(6.1)	-1.4	(4.2)	0.9	(2.0)
	%ΔHCD	-2.0	(8.6)	-6.5	(9.5)	-3.2	(10.1)
Texas unwashed	Dust	0.98	(0.03)	0.97	(0.03)	0.99	(0.03)
	Bacteria	104,009	(23,556)	107,849	(21,725)	96,210	(22,569)
	%ΔFEV$_1$	-5.1	(4.7)	-3.9	(3.5)	-4.5	(4.9)
	%ΔHCD	-12.6	(16.8)	-15.1	(10.7)	-19.9	(13.0)
Rayon	Dust	0.39	(0.02)	0.39	(0.02)	0.39	(0.02)
	Bacteria	4,001	(4,057)	4,429	(4,288)	3,252	(3,255)
	%ΔFEV$_1$	0.8	(3.8)	-2.2	(3.8)	1.98	(2.96)
	%ΔHCD	7.5	(15.2)	-3.3	(12.0)	3.5	(9.7)
Texas overspray	Dust	1.01	(0.1)	1.03	(0.10)	0.98	(0.1)
	Bacteria	81,474	(13,729)	75,420	(12,826)	83,530	(14,200)
	%ΔFEV$_1$	-3.97	(5.7)	-3.6	(7.9)	-2.3	(3.2)
	%ΔHCD	-14.1	(14.9)	-13.9	(9.9)	-6.0	(7.0)

Table 16 Exposure Related Change in Methacholine Responsiveness Using HCD.

Exposure	Overall Mean (SEM)	LS mean ≠ 0	Non-Atopic	LS mean ≠ 0	Atopic	LS mean ≠ 0
Tx Washed	0.23 (0.08)[A]	Y	0.21	N	0.25	Y
Texas Unwashed	0.76 (0.08)[B]	Y	0.67	Y	0.84	Y
Rayon	-0.05 (0.08)[C]	N	-0.14	N	0.02	N
Texas Additive	0.53 (0.07)[D]	Y	0.50	Y	0.57	N

Means within a column with different letters are significantly different ($P < 0.05$).
Positive number means an increase in responsiveness

Table 17. Change in Methacholine Responsiveness As Calculated Pre to Post Change in Slope After Exposure

Exposure	Overall	LS mean ≠ 0	Non-Atopic	LS mean ≠ 0	Atopic	LS mean ≠ 0
Tx Washed	0.14 (0.5)[A]	N	0.023	N	0.160	N
Tx Unwashed	2.39 (0.5)[B]	Y	2.60	Y	2.13	Y
Rayon	-0.06 (0.5)[A]	N	-0.29	N	-0.03	N
Tx Overspray	1.28 (0.5)[A]	Y	1.86	Y	0.68	N

Means within a column with different letters are significantly different ($P < 0.05$).

Table 18. Evaluation of Methacholine Response to Washed Cotton and Rayon

	Overall		Atopics		Non-atopics	
Exposure	Slope (SEM)	LS Mean ≠ 0	Slope (SEM)	LS Mean ≠ 0	Slope (SEM)	LS Mean ≠ 0
Texas Washed	0.30 (0.29)	N	0.42 (0.41)	N	0.20 (0.42)	N
Rayon	-0.43 (0.32)	N	-0.42 (0.40)	N	-0.41 (0.52)	N

1993 Study

Introduction

Studies in 1991 and 1992 demonstrated that both non-smoking and smoking naive atopic and non atopic subjects responded to aerosols of cotton dust with increases in non-specific bronchial reactivity. This appears to occur even when changes in ventilatory function ,e.g. FEV_1 are small or absent. These data have been summarized in the previous sections of this report. Based on this response, the question was asked: Does an exposure to an aerosol of cotton dust, which increases non-specific bronchial reactivity, also increase the response to an inert dust on subsequent exposure? The rationale for this question is as follows: If exposure to cotton dust increases non-specific airway reactivity, workers may be more susceptible to adverse effects from other exposures that occur either away from work or in other work environments. They may be at increased risk for developing chronic pulmonary impairment even in the absence of acute decrements in lung function during cotton dust exposure. The effect on airway reactivity did not appear to be closely correlated with endotoxin content of the dust in the previous studies.

The primary objectives of these studies were to assess the relative effects of the exposure to materials with differing endotoxin contents on day one and to examine differences in carry-over effects from these exposures on the response to exposures to rayon on day two. We specifically asked (a) whether the respiratory function changes differed by exposure materials; (b) whether atopic status affected change in respiratory function; (c) whether exposure material and atopic status had an interactive effect; and (d) whether the respiratory status after exposure to a relatively inert dust (rayon) differed depending on the material used for the previous days exposure.

Subject Recruitment and Selection: Non-smokers without a history of asthma or previous exposure to cotton dust were recruited by radio and newspaper advertisement. Subjects were initially screened by telephone and if qualified, invited to a follow-up screening session at the laboratory. Laboratory screening consisted of spirometry, administration of a more detailed medical and occupational questionnaire, and if not excluded by questionnaire response, evaluation of atopic status by skin test. The questionnaire asked about personal and family history of allergy and previous exposure in dusty environments. Criteria for exclusion from the study included an FEV_1 or FVC <80% predicted; FEV_1/FVC ratio <70%; previous history of exposure to cotton dust; or chronic respiratory illness.

Skin testing was performed using a sterile, disposable multiple head applicator (Multi-test^{tm}; Lincoln Diagnostics, Inc) with a battery of six inhalant allergens common to the southeastern Unites States. The allergens included: cat dander, ragweed, a southern-grass mix, pecan-hickory tree mix, mite mix, and Alternaria sp. (Greer Inc.). Histamine (1:1000 wt/vol) and saline with 50% glycerine were used as a positive and negative control respectively. The wheal associated with the histamine and saline responses were evaluated at 10 minutes after application and at 15 minutes for the specific allergens. Erythema was not used in the evaluation of atopic status. For this study, subjects were categorized as atopic if they had a positive response relative to the negative control to one or more of the specific allergens.

Experimental Design and Exposure Sequence: Subjects selected for the study were asked to participate two consecutive days a week for four weeks. Subjects were randomly divided into two groups designated as the Monday-Tuesday group or the Wednesday-Thursday group. Each week participants reported on either Monday or Wednesday and prior to entering the cardroom completed baseline spirometry. Each participant remained in the cardroom for 5 hours during which lunch was provided; restroom breaks were allowed as needed. Subjects were removed from the cardroom after 5 hours and spirometry performed. The Day One exposure was followed on Day Two (Tuesday or Thursday) with an exposure to dust generated from rayon. Prior to the follow-up rayon exposure, each subject completed pre-exposure spirometry. The testing and exposure sequence on Day Two was the same as Day One. Subjects were removed from the cardroom after 5 hours and spirometry performed.

Measurements of lung function and airway reactivity: Ventilatory function was tested with standard spirometry meeting American Thoracic Society guidelines (10). A Ohio Medical Products 822 rolling seal spirometer was used for all spirometric evaluations. Ventilatory function was compared to predicted values (11) and only those subjects whose FEV_1 was equal to or above 80% of the predicted value were retained for further evaluation for the study.

Study Cottons: Four primary exposure conditions were evaluated during the four week study. Dust was generated from four types of fiber: Rayon, Texas unwashed cotton, a blend of Texas unwashed cotton and Texas washed cotton, and a California cotton. A Texas grown cotton, predominantly 1990 crop, grade code 41 (USDA) was blended to make a 34 bale mix. A subset of the Texas cotton was washed and blended with unwashed cotton. The blend was formulated to achieve an endotoxin level that was intermediate between the Texas unwashed and the rayon. The California cotton was selected to contain a level of endotoxin that was similar to the endotoxin in the blended Texas cotton. The four exposure conditions represented four levels of endotoxin: rayon - low endotoxin; blend and California - intermediate endotoxin; and Texas unwashed - high endotoxin. The rayon was a commercially available standard textile grade fiber characterized as 1.5 denier, 1.5 inches, and bright. The target dust level for each cotton was 1 mg/m^3. The target dust level for rayon was 0.5 mg/m^3, which was the maximum achievable for the carding conditions and minimum room ventilation criteria.

Exposure Room and Environmental Monitoring: All exposures took place in an experimental cardroom at the USDA-ARS Cotton Quality Research lab in Clemson, South Carolina. The dimensions of the cardroom have been described previously (8). Chairs for subjects were situated around the perimeter of the cardroom and subjects were asked to move to a different location at hourly intervals. Dust levels were controlled by adjustment and balance of card production rates and air supply and exhaust rates (9). Dust levels were monitored using four vertical elutriators (VE) at different positions in the cardroom and with a portable continuous aerosol monitor (PPM Inc, Knoxville, TN). The particle size distribution was monitored using an Anderson Series 210 Cascade Impactor. All cottons and rayon were processed through blender-feeders and three stages of blending, opening, and cleaning on a finisher picker to prepare laps for feeding a carding machine. Carding was at 25-30 lbs/hr for the cotton and 60 lbs/hr for the rayon.

Ambient air was sampled for total viable airborne bacteria with six-stage Anderson air samplers (Anderson 2000 Inc., Atlanta, Georgia) operated at 0.028 m^3/min for 15, 30, 60, or 120 seconds. The samplers were loaded with 90 mm plastic petri plates filled with 40 ml of trypticase soy agar [30 g trypticase soy broth, 20 g agar and 5 g yeast extract per liter and cycloheximide (50 μg/ml)]. The plates were incubated for 22-30 hours at room temperature (RT;24.1±03°C).

For airborne endotoxin, VE filters were extracted in 50 ml pyrogen free water (PFW) for 1 hour in pyrogen free borosilicate glass containers. Appropriate serial 10-fold dilution were evaluated by the kinetic chromogenic modification of the Limulus amebocyte lysate (LAL) assay (BioWhittaker).

Study Design: The study design incorporated subjects randomly assigned within atopic category in two groups which were exposed on two successive days six days apart. Four weeks of measurements were obtained for each group. During each week, four measures of respiratory function were obtained for each group over a two day period, with one pre-exposure measure and one post-exposure measure obtained each day. On Day One, subjects were exposed to one of three study cottons or to rayon. All subjects were exposed to each condition over the four week study. On Day Two, all subjects were exposed to rayon. The two groups differed primarily on the days studied, with Group 1 tests conducted on Monday and Tuesday and Group 2 tests conducted on Wednesday and Thursday. The exposure levels for Day One or Day Two did not differ between groups.

Data Analysis: The preliminary analyses were conducted in three stages. First, the two randomly assigned study groups were examined for differences in respiratory function and demographic characteristics. Second, the effects of exposure material, atopic status, and their interaction on respiratory function on Day One were examined using the pre-exposure to post-exposure changes in respiratory function. Third, the effects of exposure material and atopic status were examined. Both Day Two pre-exposure respiratory levels and the exposure-related changes in Day Two respiratory function were evaluated.

For each stage of analysis, separate analyses were run on three measures of respiratory function--forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), and the ratio of FEV_1 to FVC (FEV_1/FVC). For measures that involved function at a single time point FEV_1 and FVC were measured as percent predicted and FEV_1/FVC was measured as the ratio of the actual measurements. For outcomes that involved pre-exposure to post-exposure changes, all variables were measured as a percentage change with the pre-exposure measure forming the basis.

In comparing baseline respiratory function and demographic characteristics for the two groups, two sample t-tests were used to test group differences for continuous variables, and chi-squared tests of homogeneity were used to test for differences in dichotomous measures. All of the analyses in stages 2 and 3 involved multiple outcome measures over time on the same subjects. This repeated measures design generates outcomes that are correlated rather than independent. To account for this correlation structure and to enable the use of partial data available from subjects who missed one or two test days, the analyses were conducted with the linear mixed model using the SAS procedure PROC MIXED. For all analyses, a compound symmetric covariance structure over time was assumed.

For each analysis, an initial model that included the main effects of exposure material and atopic status as well as their interaction was fit. An approximate F-test was used to test the significance of the interaction. If the interaction was found to be nonsignificant at the 0.05 level, a reduced main effects model was fit. Global approximate F-tests were then used to test for overall significance of each of the main effects. If a significant exposure material main effect was found at the 0.05 level of significance, Wald tests were then used for pairwise comparisons of the exposure material effects.

Results

Demographic data: Table 1 compares of baseline respiratory function and demographic characteristics across the two groups. Respiratory function was compared at the baseline visit and at the pre-exposure visit on the first day of exposure. All differences were nonsignificant at the 0.05 level. However, Group Two did exhibit an average FVC that was consistently lower at baseline and on the first exposure day than that of Group 1, but the difference was nonsignificant on either occasion. Because no differences were found between the two groups, all subsequent analyses were based on the combined sample.

Screening questionnaire: Five of 38 subjects gave a personal history of atopy confirmed by a physician on the questionnaire, and 4 (80%) were confirmed as atopic by skin testing (Figure 1). The PPV was 80% for the five subjects with positive questionnaire response. The NPV was 73%, signifying that over 25% of the subjects who indicated they were not atopic by questionnaire had positive skin test. Table 2 compares the results from the four years of the study. Non-atopics comprised 73% of the four years study population and 27% were designated as atopics by objective measures. In general, 81% of the subjects indicating they were atopics by questionnaire were atopic by an objective measure (either by a Rast test or by skin test). However, only 68.5% of the subjects who said they were not atopic were negative by the objective measure. These data suggest that less than 20% of the subjects responding positively to a question regarding atopic status will be non-atopic, but over 30% of the subjects that responded negatively are in fact atopic.

Skin testing: Skin testing was done with the following antigens: Cat, *Alternaria*, ragweed, southern grass mix, mite mix, and southern tree mix. Of the 38 subjects in the study, four were positive to a single antigen and nine were positive to two or more antigens. Overall there were ten responses to the grass mix, six responses to mite antigen, five to ragweed, four to *alternaria*, three to the tree mix, and two to cat. The rank order of number of positive differed between 1992 and 1993 for common antigens (Table 3). In 1992, the highest number of responses was to the mite antigen and in 1993, the largest number of responses was to the southern grass mix.

To determine if the trend may have been related to the number of positive skin test, the data were sorted by number of positive skin tests (0, 1, or >1) and baseline data compared (Table 4). The data indicates that persons with two or more positive skin tests were younger than the other groups and had lower ventilatory parameters. These differences were not analyzed statistically. Visual inspection of the baseline comparison of the two groups (Table 1) indicated that group two had lower ventilatory parameters than group one. This group contained a higher number of

atopics, 42% vs. 26%, and a larger proportion of those atopics were positive to more than one antigen (3/5 of group one and 6/8 of group two).

Pre-exposure Spirometry: Pre-exposure spirometry was evaluated prior to Day One exposures as a function of exposure material, atopic status, or their interaction. For FEV_1, FVC and the FEV_1/FVC ratio, there was no significant interaction at the 0.05 level, and the effects of atopic status were also nonsignificant. There was a significant effect of exposure for FEV_1 and FVC, but not for the ratio. Table 5 shows the model estimates and their standard errors for FEV_1 and FVC averaged across atopic status. For FEV_1, rayon (week one) was significantly different from California and Texas cotton (weeks three and four), and blend (week two) was significantly different from Texas (week four). For FVC, rayon, blend and California (weeks one-three) were significantly different from Texas (week four), and California (week three) was significantly different from rayon (week one). These data suggest there is either an effect of time or exposure on Day One pre-exposure spirometry. In our previous studies (1991 and 1992) there was a decrease in pre-exposure baseline FEV_1 over four weeks. These studies were done using a Latin square design in which subjects, divided into four groups, received one of four exposures in a randomized pattern over four weeks. This allowed the conclusion that the decrease in pre-exposure FEV_1 over the four weeks study was related to fatigue and not a carryover of exposure. In this study a Latin square design was not used, and both groups of subjects received the same exposure over the four weeks, therefore exposure and week covaried. Based on the experimental design, it is likely that the decrease observed in the FEV_1 and FVC is related to fatigue and not a carryover from the previous weeks' exposure. Another possible indicator that the association with exposure is not related to the decline is that the ratio did not change. Exposure to cotton dust is considered to result in an obstructive pattern, therefore the lack of a change in the ratio would suggest fatigue.

Pre to Post-Exposure Change in Spirometry Day One : The Day One data were also examined to assess the effect of exposure, atopic status, and their interaction on changes in spirometry from the pre-exposure to post-exposure measurement (Table 6). The interaction term was nonsignificant for all three variables, as was atopic status. The exposure effect was significant for FEV_1 and the FEV/FVC ratio, but it was not for FVC. These data would suggest an obstructive pattern. The data are presented as a percent change from pre-exposure to post-exposure. For FEV_1, the greatest change was due to the Texas cotton, followed by the washed/unwashed blend, California, and rayon. The only pairwise differences that were significant at the 0.05 level were between the Texas and California cottons and between the Texas cotton and rayon. The order of the effects was the same for ratio, but the effect of Texas cotton was significantly different than the effects of the other exposure materials based on pairwise analyses.

Day One to Day Two Exposure Interaction: The next analyses evaluated the effects of Day One exposure on Day Two pre-exposure spirometry. All interaction terms were nonsignificant, and spirometric outcomes were not significantly affected by atopic status. Two hypotheses were tested for the exposure material main effect: First, was the mean Day Two pre-exposure spirometry different for the four exposure conditions on Day One; and the second, was the difference between mean Day Two pre-exposure and mean Day One pre-exposure different

across the four Day One exposure materials. There was no significant difference in mean Day One and Day Two pre-exposure FEV_1, FVC and the ratio (i.e., the global approximate F-test). For the FEV/FVC ratio, Day Two pre-exposure means were not significantly different between exposures. However, for both FEV_1 and FVC, the mean Day Two levels were significantly different at the 0.05 level. Estimates of the Day Two pre-exposure mean FEV_1 and FVC levels (measured as a percent of predicted value) and standard errors for the four Day One exposure materials are given in Table 7. For both FEV_1 and FVC, the pattern of Day Two pre-exposure function (rayon followed by blend, California, and Texas) is the same as the Day One pre-exposure pattern, but the differences are now significant. Pairwise comparisons for FEV_1 indicate significant differences between rayon and Texas and rayon and California. For FVC, the rayon and blend are significantly different from California and Texas. Regardless of the Day One exposure, all Day Two pre-exposure FEV_1 and FVC levels were consistently lower than Day One pre-exposure levels.

The mean results for Day One and Day Two pre and post-exposures as a function of Day One exposure material are shown in figures 2 and 3 for FEV_1 and FVC, respectively. All groups show a drop in mean lung function after the Day One exposure with the drop for Texas cotton for both FEV_1 and FVC greater than the drops for the other three exposures. Overnight, all four groups show recovery, but none of the groups recover to the Day One level. All groups exhibit relatively small changes that were either slightly negative or slightly positive after the exposure to rayon on Day Two.

Day One Pre-exposure Compared to Day Two Post-Exposure: Table 8 presents the adjusted mean differences from the model for FEV_1, FVC, and their ratio. For both FEV_1 and FVC, the units of measure are percent of predicted value. Actual ratios were used for the ratio variable. For both FEV_1 and FVC, the mean change differed from 0 for at least one of the exposure materials (for FEV_1, $F=10.7$, $ndf=4$, $ddf=252$, $p=0.001$, and for FVC, $F=9.52$, $ndf=3$, $ddf=252$, $p=0.001$). However, the tests for differences in change as a function of Day One exposure material were nonsignificant (for FEV_1 $p=0.33$ and for FVC $p=0.74$). Thus, there appeared to be no effect on the level of function at the end of day two exposure which was associated with the material used for the day one exposure. For the ratio of FEV_1 to FVC the change was not significantly different from 0 for any of the four groups.

Publications:

1. Jacobs, R.R. and Thomas, Kathryn C.: the evaluation of endotoxin and glucan activity of an organic dust using glucan sensitive and insensitive endotoxin assays. In: Proceedings of the 18th Cotton Dust Research Conference, pp:350-354, 1994. Eds. L.N. Domelsmith, R.R. Jacobs, and P.J. Wakelyn, New Orleans, La., National Cotton Council, Memphis, Tn.

2. Jacobs, R. and P. Pietrowski: The Effect of Sodium Dodecyl Sulfate Versus Water Extraction on Detection of Endotoxin with the Limulus Amebocyte Lysate Assay. In: Proceedings of the 19th Cotton Dust Research Conference, 1995. Eds. R. Rylander, R.R. Jacobs, and P.J. Wakelyn, San Antonio, Tx., National Cotton Council, Memphis, Tn.

Tables and Figures

Figure 1

	Skin Test		
Questions	+	-	Total
+	4	1	5
-	9	24	33
Total	13	25	38

PPV = 80%
NPV = 73%

Figure 2 Pre and Post FEV1
Day One and Day Two Exposures

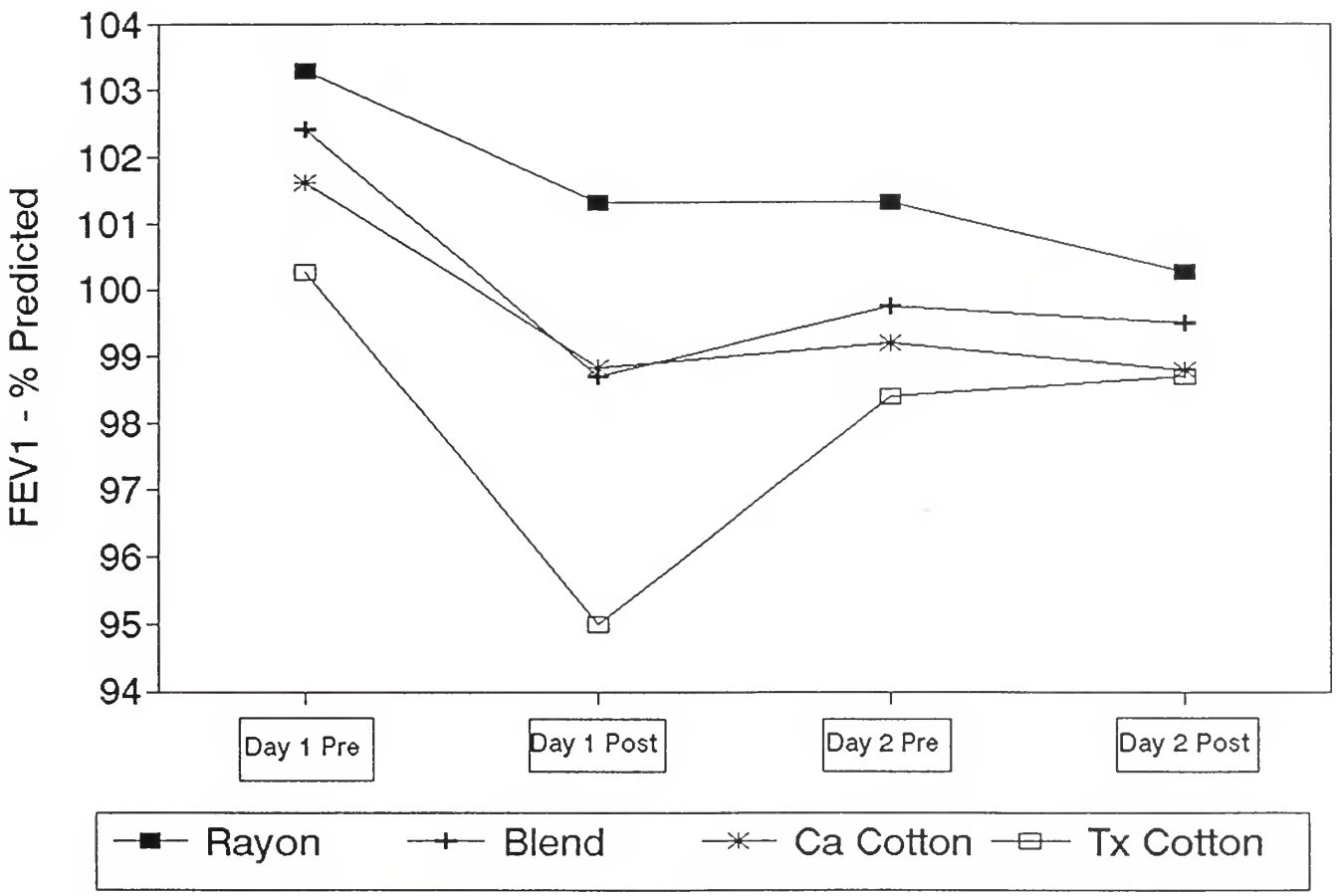

Figure 3 Pre and Post FVC
Day One and Day Two Exposures

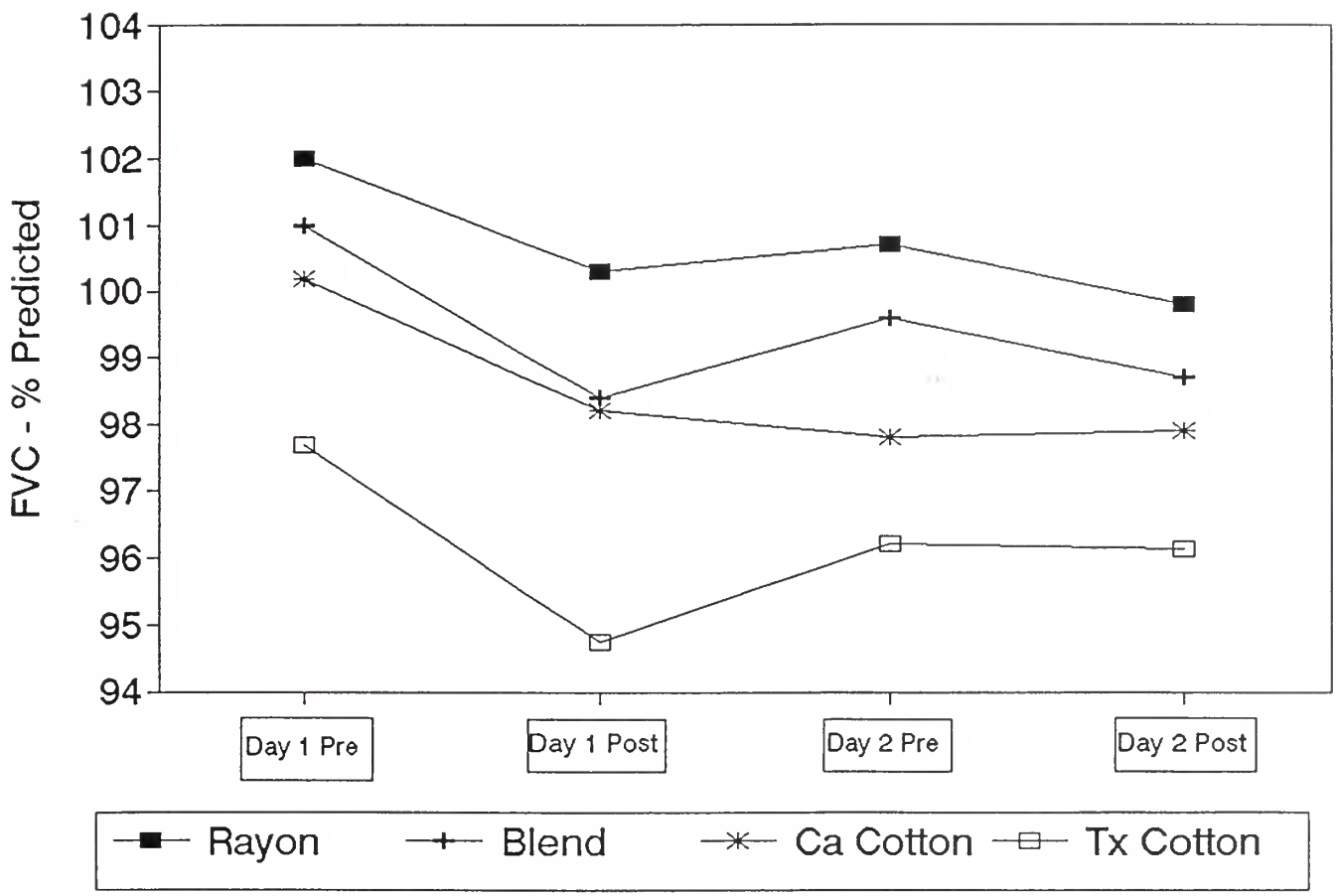

Table 1 Baseline Respiratory and Demographic Characteristics

Parameter	Group 1[a]	Group 2[a]	Test of Difference (p-value)
Baseline FEV_1, % predicted	104.1 (11.9)	103.4 (12.0)	0.87
Baseline FVC, % predicted	104.1 (12.7)	98.8 (11.2)	0.18
Baseline FEV/FVC, unitless	0.821 (0.044)	0.812 (0.18)	0.83
Day One FEV_1, % predicted	104.8 (12.0)	102.3 (12.2)	0.53
Day One FVC, % predicted	105.2 (12.2)	99.3 (12.3)	0.14
Age, years	40.7 (14.9)	39.7 (16.3)	0.84
Gender, % female	37	26	0.49
Atopic Status, % atopic	26	42	0.30

[a] Values in parentheses are standard deviations.

Table 2

	PPV		NPV		
Year	%	n	%	n	
1990[A]	100	20	84	31	51
1991[A]	44	15	65	23	38
1992[B]	100	3	52	31	34
1993[B]	80	5	73	33	38
Avg.	81		68.5		

[A]Phadiatop
[B] Skin test

Table 3 Comparison of Rank of Number of Responses for 1992 and 1993

Antigen	1992	1993
Mite	2	1
Grass Mix	1	2
Ragweed	3	3
Alternaria/Fungi	-	4
Tree Mix	5	5
Cat	4	6

Table 4 Baseline Data Sorted by Number of Positive Skin Tests

	Positive Skin Test		
	0	1	>1
Number	25	4	9
Age	42.8 (13.7)	46.3 (15.7)	30.3 (12.3)
Male:Female	5:20	2:2	5:4
Black:White	1:24	0:4	2:7
% Pred. FEV_1	103.2 (11.5)	115.0 (5.2)	100.2 (10.2)
% Pred. FVC	101.6 (11.5)	111.3 (6.0)	96.8 (8.6)
Ratio	0.83 (0.05)	0.85 (0.04)	0.77 (0.24)

Table 5 Comparison of Day One Pre-Exposure Respiratory Function

Parameter	Week	Exposure Material	Estimated Mean[1]	Standard Error	Pairwise Comparisons
FEV_1	1	Rayon	1.043^{A}	0.021	
	2	Blend	$1.034^{A,B}$	0.021	
	3	CA	$1.026^{B,C}$	0.021	
	4	TX	1.013^{C}	0.021	
FVC	1	Rayon	1.026^{A}	0.022	
	2	Blend	$1.016^{A,B}$	0.022	
	3	CA	1.007^{B}	0.022	
	4	TX	0.987^{C}	0.022	

Letters within a column indicates means are significantly different ($P < 0.05$).
[1] Values represent mean % of predicted (x 100)

Table 6 Comparison of Day One Changes in Respiratory Function

Parameter	Exposure Material	Estimated Mean[1]	Standard Error
FEV[1]	Rayon	-1.805[A]	0.93
	CA	-2.607[A]	0.93
	Blend	-3.718[A,B]	0.93
	TX	-5.625[B]	0.95
FEV/FVC	Rayon	-0.468[A]	0.61
	CA	-1.006[A]	0.61
	Blend	-1.240[A]	0.61
	TX	-2.721[B]	0.62

Letters within a column indicates means are significantly different ($P < 0.05$).
[1] Values represent mean % overexposure change

Table 7 Comparison of Day Two Pre-Exposure Respiratory Function

Parameter	Exposure Material	Estimated Mean	Standard Error
FEV_1	Rayon	1.024^{A}	0.022
	Blend	$1.008^{A,B}$	0.022
	CA	0.996^{B}	0.022
	TX	0.994^{B}	0.022
FVC	Rayon	1.012^{A}	0.022
	Blend	1.002^{A}	0.022
	CA	0.980^{B}	0.022
	TX	0.971^{B}	0.022

Letters within a column indicates means are significantly different ($P < 0.05$).
[1] Values represent mean % of predicted (x 100)

Table 8 Comparison of Change in Respiratory Function from Day One Pre-Exposure to Day Two Post-Exposure

Parameter	Day One Exposure Material	Estimated Mean Change[1]	Standard Error
FEV_1	Rayon	-3.06	0.895
	Blend	-2.88	0.829
	CA	-3.25	0.836
	TX	-1.24	0.866
FVC	Rayon	-2.17	0.717
	Blend	-2.39	0.717
	CA	-2.59	0.723
	TX	-1.50	0.749
FEV_1/FVC	Rayon	-0.0067	0.0057
	Blend	-0.0035	0.0057
	CA	-0.0050	0.0058
	TX	0.0029	0.0060

[1] Values = ([day 2 post-exposure value - day 1 pre-exposure value]/ predicted value)* 100 for FEV_1 and FVC; and day 2 post- exposure ratio - day 1 pre-exposure ratio) for FEV_1/FVC ratio.

Proposed Future Studies

The role of endotoxin in the respiratory response to cotton dust and other agricultural dusts remains to be completely resolved. Additionally, questions regarding the response to dust from synthetic fibers have recently become an issue of concern. For example, the study published by Tulane indicated that higher than expected decrements in pulmonary function were observed in the synthetic fiber textile workers. Two recent findings from data generated from the studies at Clemson suggest additional experimentation is needed to resolve these issues. First, rayon dust causes mild decrements in pulmonary function and increases in bronchial reactivity as measured by methacholine challenge. These findings corroborate the mill workers epidemiologic data (Tulane study) which suggest that synthetic fiber dust is not inert. This may be a generalized response to particulate matter. However, in the 1992/93 studies at Clemson, rayon of similar technical specifications but from different production years, had substantially different levels of endotoxin. Therefore the observed response to rayon may be either specifically particulate related, endotoxin related, or related to a combination of the two. Also some as yet undetermined agent or variable may account for the response. Second, in the 1993 study, moderate levels of endotoxin naturally occurring on a California cotton were matched with a blend of a Texas unwashed/washed cotton. Two groups were evaluated with these cottons. In one group the response (measured by acute change in PFT) to the two cottons was similar. However, in the more responsive of the two groups the response to the California cottons was less that the response to the blend cotton. These data suggest that substances other than endotoxin may be involved in the response to cotton dust.

To address the questions of the acute response to endotoxin and the acute response to particulates the following experiments are proposed.

1. Identify an extremely low endotoxin California and Texas cotton and a low endotoxin rayon.

2. Identify a total of 60 subjects (three groups of 20) that could participate in exposures one day a week for approximately 5 weeks. Subjects do not need to be selected on the basis of atopic status but they would not be excluded.

3. The exposure protocol would be as outlined in the attached table. Particulate exposures to rayon, California and Texas cottons would be at 0.5 and 1.0 mg/m^3. Then endotoxin would be added as a overspray to the picker laps and particulate exposure levels of 0.5 and 1.0 mg/m^3 be used to evaluate the response to added endotoxin.

4. The specific questions to be addressed include:

    A. Is there a particulate related response change in PFT for rayon?
    B. Does endotoxin added as an overspray to rayon alter the particulate response?
    C. Are the PFT responses to cottons from California and Texas with low levels of ambient endotoxin quantitatively different that the response to rayon without added endotoxin at two doses?

D. Is there a particulate associated response change in PFT to the California and Texas cottons?

E. Is the PFT response to the California and Texas cottons with added endotoxin quantitatively different that the response without added endotoxin?

F. Is the PFT response of the California and Texas cottons with added endotoxin quantitatively different from the response to the rayon with added endotoxin?

G. Is the PFT response of the California and Texas cottons with endotoxin quantitatively different from each other?

	Group I		Group II		Group III	
	Rayon (mg/m^3)	Rayon (mg/m^3)	Cali (mg/m^3)	Cali (mg/m^3)	Texas (mg/m^3)	Texas (mg/m^3)
No LPS	0.5	1.0*	0.5	1.0	0.5	1.0
Added LPS	0.5 + LPS	1.0 + LPS	0.5 + LPS	1.0 + LPS	0.5 + LPS	1.0 + LPS

* Groups II and III would also receive a rayon exposure at 1 mg/m^3

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Bronchial Reactivity, Atopy, and Airway Response to Cotton Dust

ROBERT R. JACOBS, BRIAN BOEHLECKE, MARIANNE VAN HAGE-HAMSTEN, and RAGNAR RYLANDER

School of Public Health, University of Alabama at Birmingham, Birmingham, Alabama, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, Department of Clinical Immunology, The Karoline Hospital, Stockholm, and the Department of Environmental Medicine, University of Gothenburg, Gothenburg, Sweden

Studies of cotton textile workers have found an association between atopy and drop in FEV_1 over a workshift. We studied the response of previously nonexposed volunteers with and without a history of mild atopy to a 5-h exposure to 1 mg/m³ of respirable cotton dust in a model cardroom. All participants were nonsmokers, had no history of asthma, and had normal spirometry. Twenty atopic subjects gave a personal history of mild respiratory allergy to pollen, dusts, or animals that had been confirmed by a physician. Thirty-two nonatopic subjects had no history of allergy. Spirometry and a methacholine challenge test were performed 1 to 2 days prior to exposure. Spirometry was repeated immediately before exposure to cotton dust; spirometry and a methacholine challenge were performed immediately after exposure. Atopic subjects showed a significantly higher mean serum IgE level to Phadiatop®, a screening test to common inhalant allergens, than did nonatopic subjects (mean percent binding, 32.1 versus 1.5; $p < 0.001$). Atopic subjects had a significantly greater mean fall in FEV_1 during exposure (8.3% versus 4.9%, $p < 0.05$). The difference in FEV_1 decline between atopic and nonatopic subjects was similar in magnitude to that reported for workshift FEV_1 declines between textile workers with and without mild atopy. Atopic subjects had significantly higher baseline methacholine responsiveness than did nonatopic subjects (26% versus 0% reaching a PD_{20}, $p < 0.0005$). After cotton dust exposure, there was a significant increase in airway reactivity in both groups (68% versus 20% reaching a PD_{20}, $p < 0.0005$). For all subjects combined baseline responsiveness was significantly related to the change in FEV_1 after exposure. These results suggest that a history of mild atopy is a predictor for decrements in FEV_1 after exposure to cotton dust in previously nonexposed healthy persons. Even in nonatopic persons a transient increase in airway reactivity may be produced by cotton dust exposure.

To understand the mechanisms and risk factors for health effects of cotton and other organic dusts, persons at increased risk must be identified. In a previous study, only 39% of the healthy volunteers screened for participation in model cardroom studies showed a significant drop in FEV_1 during 6 h of exposure to 1 mg/m³ of airborne cotton dust (1). Defining the sources of this variability and characterizing markers than can identify susceptible persons are important steps in defining adequate control strategies.

Two potential markers that may be related to the variability of the acute response to cotton dust are nonspecific bronchial responsiveness and atopic status. Several previous studies demonstrated that cotton dust can increase bronchial reactivity. Haglind and coworkers (2) studied cotton mill workers before the Monday shift and, on a later occasion, after the Monday work shift. None of 22 workers had a FEV_1 decrease above 15% when challenged with 0.1 mg methacholine before the shift, whereas 10 workers showed decreases of more than 15% after the shift. Boehlecke and coworkers (3) demonstrated an increase in nonspecific bronchial reactivity in healthy naive subjects exposed to aerosols of cotton dust. These data indicate that aerosols of cotton dust increase airway reactivity nonspecifically, which may in turn enhance the response to subsequent exposures to cotton dust or other environmental aerosols.

The relationship between preexposure bronchial reactivity and the response to cotton dust inhalation is less well defined. In a study conducted for NIOSH, volunteers were tested for bronchial reactivity, and the following week they were exposed to airborne cotton dust at 1 mg/m³ for 6 h, with measurement of FEV_1 before and after exposure (4). Although a weak trend was present, no significant association was found between the drop in FEV_1 during cotton dust exposure and the previously measured bronchial reactivity. This study was limited by the separation of several days between the cotton dust exposure and the measurement of bronchial reactivity. No post-cotton-dust exposure measurement of bronchial reactivity was carried out. Schachter and coworkers (5) reported that there was no significant difference in baseline bronchial reactivity to histamine in subjects identified as responders and nonresponders to inhaled extracts of cotton bract. However, in a subsequent study using methacholine to evaluate bronchial reactivity, baseline methacholine responsiveness was significantly greater in subjects designated as responsive to cotton bract extracts (6). The investigators concluded that the differences between the two studies may be related to differences in the response

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Correspondence and requests for reprints should be addressed to Robert R. Jacobs, School of Public Health, University of Alabama, Birmingham, AL 35294.

to histamine and methacholine or differences in the criteria used to select the study populations.

Atopy has been related to the risk for developing bronchoconstriction after an acute exposure to cotton dust. Among 226 healthy nonasthmatic adults who were exposed to 1.02 mg/m³ cotton dust, the mean FEV_1 decline was significantly larger for those who were atopic, defined as a positive prick test reaction to at least two of 10 allergens. The degree of atopy measured by the number of positive skin tests also demonstrated a significant relationship with the decrease in FEV_1 (7). Jones and coworkers (8) also found a relation between atopy and the bronchoconstriction induced by cotton dust. The same investigators examined workers in cotton seed crushing mills and evaluated atopy using skin prick tests to 10 common inhalant allergens and personal and family history of atopy (9). They reported a larger FEV_1 decline over the workshift in atopic workers unrelated to differences in demographic profiles, smoking habits, or dust levels.

To study further whether the effects of cotton dust inhalation are dependent on nonspecific bronchial hyperreactivity and to determine if there are differences in the response of atopic and nonatopic persons, we evaluated the relationship of preexisting atopy and/or bronchial hyperreactivity to the responsiveness to cotton dust in an experimental cardroom.

Methods

Subjects

Fifty-seven healthy volunteers recruited by public advertisement were selected for participation. The criteria for selection included the following: age, 18 to 35 yr; nonsmokers (< 100 cigarettes/life); no history of asthma requiring treatment or episodes of wheezing; no current use of medication; no upper respiratory tract infection within the previous 4 wk; no major medical conditions considered to significantly increase the risk of participation in an exposure study; no previous occupational exposure to organic dusts; no chronic respiratory condition including chronic bronchitis; FEV_1 not less than 80% of the predicted value. Women were excluded if pregnant or currently nursing. Each potential participant was screened with a brief questionnaire by telephone to determine if any of the above criteria were present. Eligible subjects were asked to come to the laboratory where a more detailed questionnaire was administered.

Twenty of the 25 subjects reporting a personal history of atopy indicated that their atopic status had previously been confirmed by a physician. These were classified as the atopic group. The five subjects giving a nonphysician confirmed history of atopy were eliminated from further analysis. There was a higher proportion of men in the confirmed atopic population, but there was no difference in age between the two groups.

Measurements of Lung Function and Airway Reactivity

Ventilatory function was tested with standard spirometry meeting American Thoracic Society guidelines (10). An Ohio 822 rolling seal spirometer (Ohio Medical Products, Atlanta, GA) was used for all spirometric evaluations. Ventilatory function was compared with predicted values (11), and only those subjects whose FEV_1 was equal to or above 80% of the predicted value were retained for further evaluation for the study.

A test of bronchial reactivity was performed with graded doses of inhaled methacholine using a modification of the method of Yan and coworkers (12). Solutions were inhaled using a Pari-Boy nebulizer modified to deliver 0.003 ml per activation. Baseline spirometry was performed and then repeated after three breaths of normal saline.

No subject had a drop of 10% or more in FEV_1 after saline inhalation. A single maximal spirometric maneuver was then performed 3 min after each dose of methacholine. A second effort was performed only if the first was technically invalid. The initial dose was one breath of a 6.25-mg/ml concentration. Subsequent inhalations were given 5 min apart. Methacholine inhalations were continued until the FEV_1 had fallen by 20% from the postsaline value or the highest dose of six breaths of 50 mg/ml had been given. After completion of the test, subjects experiencing symptoms from the methacholine challenge were offered a standard bronchodilating agent (albuterol) by inhalation to relieve the symptoms.

Results from methacholine challenge were evaluated in two ways. For the first method, the percent change in FEV_1 from the postsaline value was calculated at the highest dose of methacholine common to both preexposure and postexposure for each subject. For the second method, the dose response slope was calculated for each challenge using least-squares linear regression of the percent change in FEV_1 from the postsaline value against the log of the cumulative inhaled dose of methacholine. The fitted line was forced to cross the point of no fall in FEV_1 at zero dose of methacholine.

Nasal Lavage

Preexposure nasal lavage was performed immediately before entering the exposure chamber using a modification of the technique of Graham and coworkers (13). Sterile phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (GIBCO, Grand Island, NY) was warmed to 37° C, and 5 ml was instilled in each nasal cavity using a 10-ml needleless syringe. The saline was held in the nasal cavity by palatal pressure for 10 s and then dispelled forcefully in a sterile plastic specimen cup.

The volume of lavage recovered was recorded and transferred to a 15-ml graduated polypropylene centrifuge tube. An equal volume of Sputolysin (Calbiochem, San Diego, CA) was added to each tube, vortexed for 1 min, and maintained at room temperature for 15 min. Each sample was then centrifuged at 1200 rpm for 10 min at 4° C, and the supernatant was discarded. The cell pellet was resuspended in 1 ml of PBS. Cells were stained using Türk's solution and counted using a hemocytometer. For counting purposes, samples were assigned a value of < 1,000 cells/ml if there was one or no cells detected in the hemocytometer grid. Because of the wide variability in the data and to satisfy the equal variance and normal distribution assumptions, the cell counts were converted to natural logarithms.

Definition of Atopy

Subjects were categorized as atopic based on a personal history of atopy as determined by questionnaire. Subjects were asked if they had hay fever or seasonal allergies. Those responding positively were asked if this had been confirmed by a physician. An aliquot of venous blood was collected for later confirmation of atopic status by a cumulative RAST test to measure serum IgE antibodies against a mixture of relevant inhalant allergens present on a solid phase (Phadiatop®, Cap system; Pharmacia Diagnostics AB, Uppsala, Sweden). The results are usually reported by the manufacturer as positive or negative relative to laboratory-determined cutoff values. For these studies, the absolute counts for each subject were reported, and the percent binding relative to the maximal possible binding was calculated. This provides a quantitative measure of the amount of IgE antibody present, with a larger percent binding indicative of a higher concentration. The Phadiatop has shown high specificity and sensitivity for identification of atopic patients (14).

Cotton Dust Exposure

After spirometry, the subjects entered an exposure room 15 × 13 × 10 feet maintained at approximately 22° C and 50% relative humidity with a controlled inflow and outflow of conditioned, filtered air. Cotton dust was generated from a commercial carding machine similar to ones used in a nonautomated textile mill. Cotton typical for commercial textile plants (Memphis territory cotton classified as Strict Low Middling, Grade 41) was provided in preblended picker laps by the USDA. Dust levels were monitored continuously with an optical particle measuring device (PCAM; PPM Inc., Knoxville, TN) and gravimetrically using millipore polyvinyl chloride filters (Millipore Corp., Bedford, MA) and vertical elutriators (VE) with 15-micrometer mass median aerodynamic diameter cutoff size. Particle size distribution was determined by an eight stage cascade impactor (Anderson). The amount of dust on the VE filters was weighed, and the endotoxin was determined as previously described (15).

Participants were allowed to move freely throughout the room except near the carding machine. Participants remained in the exposure room for 6 h except for restroom breaks and a 1-h lunch break. After 6 h, the

TABLE 1

CHARACTERISTICS OF THE STUDY POPULATION*

	Atopic Subjects	Nonatopic Subjects	Nonconfirmed Atopic Subjects	Total
Subjects, n	20	32	5	57
Male, n	17	19	2	38
Mean age, yr	23.3 (3.5)	24.7 (3.7)	22 (1.6)	23.8 (3.7)
MEAN FEV_1, % pred	103 (7)	111 (8)	119 (14)	110 (9.4)
Mean % FEV_1/FVC	86 (7)	84 (7)	92 (3)	85 (7)
Mean Phadiatop, % binding	32.4 (19.4)	1.5 (1.7)	14.1 (13)	13.4 (19.0)
Mean dust level, mg/m³	1.2 (0.1)	1.1 (0.2)	1.1 (0.2)	1.1 (0.2)

* Values in parentheses are standard deviations

exposure was terminated, and each subject was asked to repeat spirometry, methacholine challenge, and nasal lavage.

The use of human subjects for all exposure protocols was approved by the institutional review boards of the University of Alabama at Birmingham, the University of North Carolina, and the University of Gothenburg. Informed and written consent was obtained from each subject.

Statistical Analysis

Spirometry and methacholine data were evaluated using paired *t* test for preexposure to postexposure changes within subjects and independent *t* tests for comparisons between atopic and nonatopic subjects. Multiple linear regression was used to evaluate the relationship between continuous predictor variables and spirometric response variables. Cellular differences between preexposure and postexposure lavage were assessed by paired *t* tests. Differences were considered statistically significant at the $p \leq 0.05$ level. All analysis were performed using a standard statistical software package (SOLO; BMDP Statistical Software, Los Angeles, CA).

RESULTS

Subject Demographics and Exposure Conditions

Demographic data, baseline spirometry, and atopic status of the subjects are given in table 1. Nonatopic subjects had a significantly higher baseline FEV_1 in percent of predicted than did atopic subjects. Although there were only three women in the atopic group, they accounted for much of the difference between atopic and nonatopic subjects. The baseline FEV_1 in percent of predicted for the three women in the atopic group was 102%. For the 13 nonatopic women the mean FEV_1 was 113% of predicted. The difference was not significant for men separately. The percent binding of the Phadiatop ranged from 0.56 to 79.3 for the entire group. The mean for the atopic subjects was significantly larger than that for the nonatopic subjects (32.4 and 1.5, respectively).

The relation between questionnaire and Phadiatop data for atopic status is reported in table 2. The sensitivity of physician-confirmed atopy reported on questionnaire, using Phadiatop as the standard, was 80%. The positive predictive value (PPV) was 100%, and the negative predictive value was 84%.

Time-weighted average concentrations of aerosols of cotton dust in the room ranged from 0.92 to 1.49 mg/m³ on the exposure days. The mean exposure for days when atopic subjects were examined was 1.18 mg/m³, which was not significantly different from that for nonatopic subjects (1.11 mg/m³). The average value of airborne endotoxin was 1.02 μg/m³. Gravimetrically, 80% of the dust particles were less than 4 μm aerodynamic diameter in size.

Spirometry and Methacholine Responsiveness

There was a significant fall in FEV_1 of −6.0% during dust exposure for the overall group ($p < 0.005$) (table 3). However, the subjects with confirmed atopy had a significantly larger decrease in preexposure to postexposure FEV_1 than did the nonatopic subjects. Significant falls in FEF_{25-75} and FVC also occurred for both atopic and nonatopic subjects, and again the atopic subjects had significantly greater declines than did the nonatopic subjects.

Before exposure to cotton dust, the atopic subjects were significantly more responsive to methacholine with a slope of approximately twice that of the nonatopic subjects (table 4). Comparison of the slopes for men separately also demonstrated a significant difference between the preexposure responsiveness of the atopic and nonatopic subjects despite no significant difference in baseline FEV_1 between these groups. These differences were confirmed when the methacholine challenge test results were expressed as the percentage of those reaching a PD_{10} or PD_{20}, (i.e., the cumulative dose of methacholine causing a 10% or 20% fall in FEV_1, respectively). None of the nonatopic subjects reached a PD_{20} preexposure compared with 26% of the atopic subjects.

The postexposure methacholine response slope increased significantly from preexposure values for both atopic and nonatopic subjects (2.7- and 2.8-fold, respectively). The relative hyperresponsiveness of the atopic subjects persisted, with a postexposure slope twice as large as that of the nonatopic subjects ($p < 0.003$). After the exposure, 20% of the nonatopic subjects reached a PD_{20} compared with 68% of the atopic subjects.

The change in methacholine responsiveness after exposure was also demonstrated by the percent fall in FEV_1 at the highest dose of methacholine common to the preexposure and postexposure challenges for each subject. The mean change in FEV_1 at the highest common dose increased from −6.6% before exposure to −19.2% after exposure for atopic subjects, whereas for the nonatopic subjects, these values were −5.3 and −11.3%, respectively.

Exclusion of five subjects with negative histories but positive Phadiatop results for atopy and one subject lacking a Phadiatop value allowed comparison of subjects with both positive history and Phadiatop with those with neither. The drop in FEV_1 after exposure was also higher for this atopic group (8.5% versus 5.5%), but this difference was only of borderline statistical significance ($p = 0.06$).

TABLE 2

EVALUATION OF ATOPY BY QUESTIONNAIRE AND PHADIATOP TEST

	Phadiatop*	
Questionnaire	Nonatopic	Atopic
Nonatopic	26	5
Atopic	0	20

* One subject lacking a Phadiatop value.

TABLE 3

MEAN PERCENT CHANGE IN SPIROMETRIC VALUE AFTER EXPOSURE*

	Overall (n = 52)	Atopic Subjects† (n = 20)	Nonatopic Subjects† (n = 32)	p Value
FEV_1	−6.0 (0.6)	−8.3 (1.3)	−4.9 (0.7)	< 0.005‡
FEF_{25-75}	−10.5 (1.2)	−13.4 (2.5)	−8.6 (1.5)	< 0.05‡
FVC	−3.4 (0.5)	−5.2 (0.8)	−3.0 (1.0)	< 0.02‡

* Values in parentheses are standard errors of the mean.
† Defined by questionnaire (atopic = physician-confirmed).
‡ Atopic versus nonatopic.

TABLE 4

METHACHOLINE BRONCHOPROVOCATION RESULTS

	Preexposure	Postexposure	p Value*
Atopic subjects, n = 19†			
Slope‡ (SEM)	−0.034 (0.007)§	−0.091 (0.01)§	< 0.0005
Those reaching ⩾ 10% fall in FEV_1, n (%)	11 (58)§	18 (95)§	< 0.004
Those reaching ⩾ 20% fall in FEV_1, n (%)	5 (26)§	13 (68)§	< 0.005
Change in FEV_1 at HCDǁ, % (SEM)	−6.6 (0.01)	−19.2 (0.02)	< 0.0001
Nonatopic subjects, n = 30†			
Slope, (SEM)	−0.016 (0.002)	−0.044 (0.007)	< 0.0005
Those reaching ⩾ 10% fall in FEV_1, n (%)	5 (17)	15 (50)	< 0.003
Those reaching ⩾ 20% fall in FEV_1, n (%)	0 (0)	6 (20)	< 0.03
Change in FEV_1 at HCDǁ, % (SEM)	−5.3 (0.01)	−11.3 (0.01)	< 0.0001

* Preexposure to Postexposure comparison.
† One atopic and two nonatopic subjects lacked valid methacholine data.
‡ Slope is percent change in FEV_1 on log cumulative methacholine dose.
§ Significantly different from nonatopic subjects ($p < 0.05$).
ǁ Highest dose of methacholine common to preexposure and postexposure challenge.

When atopy was defined by Phadiatop results alone, disregarding history, the difference in FEV_1 change after exposure between atopic and nonatopic subjects (7.2% versus 5.5%) was not statistically significant.

Forty-nine subjects had valid preexposure and postexposure methacholine challenge tests. The drop in FEV_1 after dust exposure for those with preexposure methacholine dose-response slopes greater than the median value was significantly larger than that for those with slopes below the median value (8.4% versus 4.5%, $p < 0.006$). For all subjects combined there was a significant linear relationship between baseline methacholine slope and change in FEV_1 after exposure ($p < 0.002$).

Nasal Lavage

Nasal lavage was completed on 52 subjects (20 physician-confirmed atopic and 32 nonatopic). Valid postexposure lavage was obtained on only 28 of 32 nonatopic subjects. Overall, the mean volume recovered for the preexposure lavage was 6.5 ml compared with 6.4 for postexposure lavage. The range for total cell counts per milliliter of nasal lavage preexposure was < 1,000 to 2.9×10^5. The majority of subjects (77%) had preexposure lavage counts of 10,000 cells/ml or less, and 19% had counts of < 1,000 cells/ml; 58% had counts ranging from 1,000 to 10,000, and 23% had counts in excess of 10,000 cells/ml. The mean preexposure value for nonatopic cell count (1.4×10^4) was less than the atopic cell count (2.7×10^4), although the difference was not statistically significant.

For the subjects combined, there was a significant increase in the number of cells after exposure to cotton dust. The mean cell count increased from 1.8×10^4 cells/ml before exposure to 3.3×10^4 after exposure ($p < 0.05$). Nonatopic subjects showed a highly significant increase in the number of cells ($p < 0.001$), whereas atopic subjects had no significant increase.

Subjects were divided into three categories according to preexposure cell lavage data: < 1,000, 1,000–10,000, and > 10,000 cells/ml (figure 1). For nonatopic subjects, those in the first two categories had a significant increase in cell number after cotton dust exposure (n = 28; $p < 0.001$). For atopic subjects, none of the preexposure categories showed a significant increase in cell

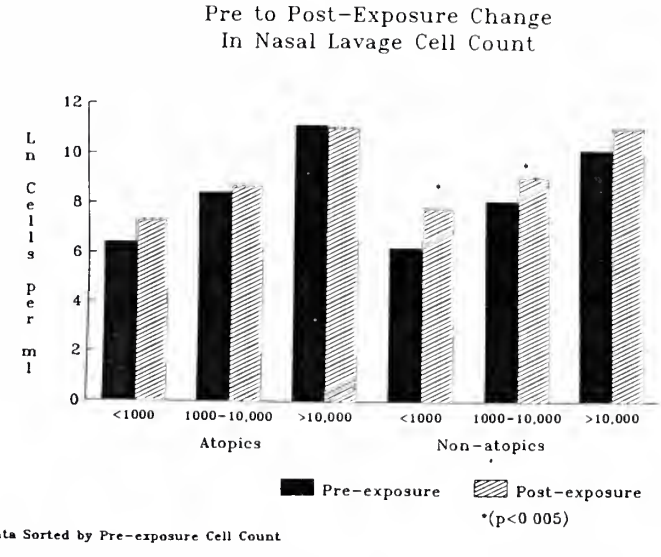

Figure 1. Change in nasal lavage cell count induced by exposure to cotton dust among atopic and nonatopic subjects.

number after cotton dust exposure (n = 20). For atopic subjects, the mean change in FEV_1 during exposure was not related to the preexposure category cell count (−6.1, −9.7, and −5.3%). For nonatopic subjects, there was a trend for greater falls in FEV_1 as the preexposure cell count increased (−3.3, −5.3, and −5.4%), but this did not reach statistical significance.

DISCUSSION

Acute decrements in lung function after several hours exposure to cotton dust have been demonstrated previously in textile mill workers and in volunteers in experimental studies (1, 16, 17). This study confirms that significant acute declines in several measures of ventilatory function can occur in young healthy nonsmokers after exposure to about 1 mg/m^3 of respirable cotton dust.

Subjects with mild respiratory allergy by history had significantly larger falls in ventilatory function even though they had not experienced recent symptomatic episodes of their allergies or had prior exposure to cotton dust. This is in agreement with results from previous studies where atopy defined by skin test has been associated with a fall in FEV_1 after dust exposure in cotton seed mill workers (8) and in volunteers (7), although almost half of the latter group were current or exsmokers, and many had previous textile mill exposure.

The atopic subjects as defined by history had a higher baseline nonspecific bronchial responsiveness than did the nonatopic subjects, judging by the steeper dose-response slope and the greater proportion reaching a 10 or 20% fall in FEV_1 after methacholine. Although a lower ventilatory function prior to inhalation challenge could bias results toward this finding (18), the difference in FEV_1 between atopic and nonatopic men was small and therefore not likely to account for the observed difference in responsiveness. Also, the participants with baseline methacholine slopes greater than the median had the same mean baseline FEV_1 percent predicted as those with slopes below the median value. These atopic subjects also had a significantly higher level of specific IgE by a multiallergen mix RAST test for several common aeroallergens. Serum level of total IgE has been associated with increased bronchial responsiveness among middle-aged and elderly men, especially those who smoked (19). However, smoking was not an independent predictor of responsiveness in those who were skin test negative, suggesting that atopy was the more important factor.

Both atopic and nonatopic subjects developed significantly higher bronchial reactivity during the exposure to cotton dust. This is also in agreement with previous studies where an increased bronchial reactivity was found after exposure to cotton dust (2, 4).

Information on bronchial reactivity is also available from studies of other organic dusts. Mink and coworkers (20) found an increased bronchial reactivity in grain handlers matched for age and specific airway conductance with control subjects. The grain handlers reported more respiratory symptoms despite a higher prevalence of atopy by skin testing and serum IgE level in the control subjects. A variation in bronchial reactivity with occupational exposure has been demonstrated by Hensley and coworkers (21), who examined bronchial reactivity in 29 workers before, during, and after the wheat harvest season. The number of persons in whom a standard histamine dose caused a decrease in FEV_1 of 20% increased during the summer harvest season and returned to a lower number during the winter.

The present study shows that the airway reactivity of the nonatopic subjects increased acutely in response to a 5-h exposure to cotton dust, although not to the same absolute level as that of the atopic subjects. This was demonstrated by the dose-response slope method of analysis as well as by the proportion of subjects reaching a 10 or 20% fall in FEV_1 during the challenge procedure.

The importance of the increased nonspecific airway reactivity induced by cotton dust in the production of chronic airway disease in persons exposed over longer periods is uncertain. Increased bronchial reactivity is associated with airway inflammation (18), and these results suggest that both atopic and nonatopic subjects experience an inflammatory airway response to cotton dust. Increased airway reactivity has also been associated with reduced ventilatory capacity in population samples even after age and smoking have been controlled (22). Other exposures such as general or indoor air pollutants may interact to produce significant chronic effects in persons with increased airway reactivity induced by workplace exposure to organic dusts. If so, atopic persons may be at greater risk for chronic effects even when workplace airborne dust concentrations are maintained at levels safe for nonatopic persons. If workers at increased risk could be accurately identified, special attention could be given to exposure limitation for these persons.

The *in vitro* RAST test used to identify atopic subjects in this study has a high sensitivity and specificity for clinically diagnosed inhalant allergy (14, 23, 24), but by itself it was not a significant predictor of response to cotton dust. Increased baseline methacholine responsiveness was significantly associated with both an atopic history and an increased FEV_1 response to dust exposure. Although in general persons with increased IgE levels would be expected to have higher methacholine responsiveness, other factors such as viral upper respiratory infections and recency of exposure to specific antigens can alter the relationship between IgE level and responsiveness. Although we excluded persons with recent viral infections or organic dust exposures, other undocumented factors may have weakened the relationship between IgE levels and methacholine responsiveness in the study group. This could account for the finding that a history of symptoms considered consistent with atopy by a physician was a better predictor of responsiveness to dust than were IgE levels alone. Further study is needed to define the relationship between atopy and responsiveness to cotton dust and whether a history consistent with respiratory allergies is useful to define persons at increased risk for adverse effects from chronic exposure.

The influx of cells in the nasal cavity in response to cotton dust is in agreement with results from other studies using both animals and bronchoalveolar lavage of human subjects, which have demonstrated that aerosols or extracts of cotton dust cause the influx of cells to the airways (25, 26). Other studies have demonstrated that exposure to oxidant gases causes a marked inflammatory cell influx in the nose in both normal (13, 27) and asymptomatic allergic subjects (28), supporting the conclusion that this reaction is a nonspecific response to an inflammatory agent.

Holopanien (29) observed that subjects with atopic rhinitis had greater numbers of PMNs in nasal smears than did a comparable group without symptoms. The average baseline counts seen in this study were intermediate to those reported for nonatopic and asymptomatic allergic subjects (13, 28). Histopathologic studies have demonstrated marked inflammation in the airways of asthmatics with infiltration of inflammatory cells, particularly eosinophils, and disruption of airway epithelium. Similar but less severe pathologic changes have been observed in subjects with asymptomatic hyperresponsiveness. The lack of a significant increase in cells in the atopic subjects could be due to a chronic state of increased inflammation compared with that in nonatopic

subjects as evidenced by the former group's higher preexposure cell count.

In summary, the study demonstrated that exposure to an aerosol of cotton dust caused acute decrements in FEV_1, which were more pronounced in those with an atopic history. Baseline methacholine responsiveness was also a predictor of responsiveness to cotton dust, whereas serum IgE antibodies against a mixture of relevant inhalant allergens alone were not. The exact relationship between atopy, airway reactivity, and responsiveness to cotton dust cannot be determined from this study. Exposure to cotton dust also increased the responsiveness to methacholine, again more pronounced in atopic subjects but also found in nonatopic subjects. This suggests that even nonatopic subjects may be at risk for developing hyperresponsiveness of the airways if chronically exposed to organic dusts. Thus, airway hyperresponsiveness appears to be both a risk factor for and an effect of response to cotton dust.

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CELLULAR INFLUX IN NASAL LAVAGE OF HUMANS EXPOSED TO COTTON DUST

Robert R. Jacobs[1], Brian Boehlecke[2], and Ragnar Rylander[3]
[1]Associate Professor, Department of Environmental Health Sciences, Graduate School of Public Health, University of Alabama at Birmingham, [2]Professor, Pulmonary Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, [3]Professor, Department of Environmental Medicine, University of Gothenburg, Sweden

Abstract

Non-smoking human volunteers aged 18-35 were exposed to aerosols of 1 mg/m^3 cotton dust for 5 hours in an experimental cardroom. Spirometry and nasal lavage were done prior to exposure and repeated immediately after exposure. Subjects were categorized as atopic or non-atopic based on their response to a screening questionnaire. Atopic status was confirmed by a quantitative serologic test. The group mean decline in FEV_1 was -6% for all subjects. Confirmed atopics had a significantly larger decline in FEV_1 (-8.3%) than non-atopics (-4.9) ($p<0.001$). Cell counts in post-exposure nasal lavage increased an average of 212% over pre-exposure lavage counts for all subjects ($P=0.05$). For non-atopics there was a significant increase in cell counts (319%) from pre-to post-exposure nasal lavage ($p<0.01$). For atopics there was no difference in cell counts between pre-and post-exposure lavage. These data indicate that atopics are more responsive to aerosols of cotton dust than non-atopics when using airflow measurements as a marker of reaction. A similar increase in responsiveness was not observed in atopics when using change in cell count as a marker of reaction, however cell number may be an indicator of baseline inflammation and the elevated cell counts in pre-exposure lavage of atopics may be a marker of increased airway reactivity which would result in increased responsiveness.

Introduction

In vivo studies using both animal and human exposures have documented the cellular and biochemical effects of cotton dust on the respiratory tract (1,2). Among the demonstrated responses is that cotton dust induces the recruitment of neutrophils to the airways in a variety of animal species, including hamsters, rabbits, and guinea pigs (3-5). This response has also been documented in humans using lung lavage from volunteers exposed to extracts of cotton dust (6). The procedures used to assess the cellular and biochemical effects of exposure to cotton dust in human subjects or animal species exposed in vivo are invasive, highly variable, and in animal studies generally require the destruction of the animal. Alternative methods that are less invasive and traumatic, that can be applied to large numbers of subjects, and that accurately reflect the effects of exposure are needed to assess the effects of cotton dust or other environmental or occupational toxins in human subjects. Nasal lavage is a technique that has been used in immunologic research to assess the immune response to viral infection, nasal rhinitis, and the effect of allergen exposure (8).

More recently this technique has been adapted to assess the effects of exposure to ozone on cell recruitment to the nasal mucosa and changes in the biochemical content of the lavage fluid (8,9,10). The technique is simple, quantitative and may provide an effective means to assess the cellular and biochemical events that take place distally in the lung after exposure to toxic aerosols.

Both nasal swabs (11) and nasal washings (12) have been used to assess the response to aerosols of cotton dust. These studies demonstrated the recruitment of neutrophils to the nasal mucosa following exposure to cotton dust but failed to demonstrate a dose-response relationship between cell number and change in FEV1 (food expiration value in one second FEV_1) The failure to demonstrate a dose-response relationship may reflect either the lack of a relationship in the two responses or the semi-quantitative nature of these procedures. Furthermore the subjects used in these studies were not stratified by variables known to influence the response to cotton dust such as smoking and atopy. To assess the nasal lavage procedure, samples were collected from non-smoking atopic and non-atopic volunteers before and after exposure to aerosols of cotton dust. Lavage samples were centrifuged and the cell pellet resuspended in phosphate buffered saline and counted by microscopy. Comparison of total cell counts were made before and after exposure for each group of subjects. Comparisons were made between changes in cell number and spirometric parameters.

Methods

Subject Selection

Fifty-seven healthy volunteers recruited by public advertisement were selected for participation. The criteria for selection included the following: Age 18-35 of both sexes; Non-smokers (<100 cigarettes/life); No history of asthma requiring treatment or episodic wheezing; No current use of medications for breathing or which are felt to affect bronchial reactivity; No recent upper respiratory tract infection (within 4 weeks); No major medical conditions considered by the co-investigators to significantly increase the risk of participation in an exposure study (eg. angina pectoris); No previous occupational organic dust exposure; No chronic respiratory condition including chronic bronchitis; Pregnant women or those currently nursing were excluded; and FEV_1 less than 80% predicted value.

Each potential participant was screened with a brief questionnaire by telephone to determine if any exclusion criteria were present. Subjects meeting the prescreening criteria were asked to come to the laboratory where a more detailed questionnaire was administered by the technical staff.

Subject Screening

Ventilatory function tested with standard spirometry was used to measure vital capacity and FEV_1. Ventilatory function was compared to predicted values (13) and only those subjects whose FEV_1 was equal to or above 80% of the predicted value were retained for further evaluation for the study. Subjects were categorized as atopic based on a personal or family history of atopy as determined by questionnaire. An aliquot of blood was collected for later confirmation of atopic status. Blood samples were evaluated using a modified RAST test, the Phadiatop (Pharmacia Diagnostics AB, Uppsala, Sweden). This test evaluates serum IgE antibodies against a balanced mixture of relevant inhalant allergens. Data are reported by the manufacturer as positive or negative relative to a laboratory determined cutoff value. For these studies the absolute counts for each subject were reported and the investigators calculated the percent binding relative to the maximum possible binding. This provides a quantitative estimate of atopy with a larger percent binding indicative of a stronger response. The Phadiatop has shown high specificity and sensitivity for identification of atopic individuals (14).

Subject Exposure

One or two days prior to exposure, each subject performed methacholine challenge and nasal lavage to establish baseline values. On the exposure day, subjects reported at different times to allow for sequential completion of the exposure and follow-up test. Prior to exposure each subject performed spirometry and then entered the exposure area which was a 15 x 30 x 10 ft room with a controlled inflow and outflow of conditioned filtered air with a temperature of approximately 70 F and 50% relative humidity. Cotton dust was generated from a commercial carding machine similar to ones used in a typical textile mill. The cotton for exposures was selected to be representative of a "typical cotton" used in the US and Swedish textile industries. The cotton was provided as part of a cooperative agreement by the Cotton Quality Research Laboratory of the Agricultural Research Service of the United States Department of Agriculture (USDA-ARS) in Clemson, South Carolina (15). The cotton selected for the study was harvested in the 1989 growing season and classed as grade 41, a Strict Low Middling,

Memphis territory cotton. All cotton for the study was blended, formed into picker laps (blended rolls of cotton that can be mounted on the carding machine) and shipped to Sweden. Preliminary studies were done to determine the stability of dust concentrations in the room at selected carding conditions. Dust concentrations were maintained constant at 1 mg/m^3 ± 0.1 mg/m^3 by dilution ventilation. Dust levels were monitored continuously with an optical particle measuring device (PCAM, PPM Inc. Knoxville, Tn) and gravimetrically using vertical elutriators.

Participants were allowed to move freely throughout the room except near the carding machine. Barriers were placed around the carding machine and an automatic cutoff device installed to safeguard against injury. Subjects were also instructed concerning safe practice around a carding machine. Hearing protection was provided for all subjects entering the cardroom. A technician was in an adjoining room at all times and subjects were instructed to report any symptoms. Participants remained in the exposure room for 5 hours except for restroom breaks and a one hour lunch break.

After 5 hours, the exposure was terminated and each subject asked to repeat spirometry, methacholine challenge, nasal lavage and answer a brief questionnaire about symptoms. Subjects were given a second questionnaire about symptoms to complete 24 hours after the exposure.

Nasal Lavage

The technique of Graham et. al. was adapted to collect samples for analysis (10). Sterile phosphate buffered saline, without Ca and Mg (Gibco) was warmed to 37 C and 5 ml instilled in each nasal cavity using a 10 ml needleless syringe. The saline was held in the nasal cavity by palatal pressure for 10 seconds and dispelled forcefully in a sterile plastic specimen cup.

The volume of lavage recovered was recorded and transferred to a 15 ml graduated polypropylene centrifuge tube. An equal volume of Sputolysis (Cal-Biochem) was added to each tube and vortexed for 1 minute and maintained at room temperature for 15 minutes. Each sample was then centrifuged at 1200 rpm in a clinical centrifuge for 10 minutes at 4 C and the supernatant discarded. The cell pellet was resuspended in 1 ml of PBS and counted using a hemocytometer. Cells were stained using Turk's stain. For counting purposes samples were assigned a value of <1000 cells/ml if there was one or no cells detected in the hemocytometer grid.

Data Analysis

Because of the wide variability in the data and to satisfy the equal variance and normal distribution assumptions, the cell counts were converted to natural logs. Cellular differences between pre- and post-exposure lavage were assessed by paired t-test. A P value of <0.05 was considered significant.

Results and Discussion

Study Population

Demographic data for the 57 subjects that participated in the studies are shown in Table 1. For data analysis subjects were categorized as atopic or non-atopic by questionnaire. Twenty of the 25 subjects indicating a personal history of atopy reported their atopic status had previously been confirmed by a physician. The five subjects giving a non-physician confirmed history of atopy were eliminated from further analysis of the data. Subjects identified as non-atopic reported no personal history of atopy. There was no difference in the ages of the different categories of the study population, however there were significantly more males in the confirmed atopic population. This distribution of males and females in the confirmed atopics was accounted for in further data analysis. Non-atopics had a significantly higher baseline FEV_1 than confirmed atopics. When these populations were stratified by gender the difference in baseline FEV_1 for males disappeared. There were only 3 females in the confirmed atopic group so no comparison between groups was made. Atopic status as determined by questionnaire was confirmed by a quantitative serological test, the Phadiatop®. Percent binding ranged from 0.56 to 79.3 % positive control. For confirmed atopics the mean value (32.4%) was significantly larger than for the non-atopics(1.5%, $p<0.01$). Three of the 5 subjects reporting a personal history of atopy unconfirmed by a physician were positive by Phadiatop®. The mean Phadiatop® value for non-doctor confirmed atopics was intermediate between the non-atopics and doctor confirmed atopics (14.1%). These data suggest that in this population the questionnaire was a reliable tool for the identification of persons at increased risk (doctor confirmed atopics) for an elevated spirometric response from exposure to cotton dust. If a questionnaire could be used to identify workers at increased risk for a response to cotton dust then the steps necessary to safeguard the health could be justified more effectively to management and implemented more rapidly.

Spirometry

Subjects were exposed to aerosols of cotton dust ranging from 0.92-1.49 mg m^{-3}. Gravimetrically, 80% of the dust generated was less than 4 μm aerodynamic equivalent diameter. Mean exposure for confirmed atopics (1.18 mg m^{-3}) was not significantly different than exposure levels for non-atopics (1.11 mg m^{-3}).

For all subjects the mean change in FEV_1 after exposure to cotton dust was significantly different from zero (Table 2). Significant changes were also observed for the FEF_{25-75} and the FVC. When stratified by atopy, both confirmed atopics and non-atopics showed an acute response to the dust that was significantly different than zero. The confirmed atopics declined in FEV_1 an average of -8.3% while non-atopics declined an average of -4.9%. The difference between atopics and non-atopics was statistically significant for FEV_1 and the other spirometric parameters of FEF 25-75 and FVC. These data are similar to results reported for both naive subjects (16) and cotton textile workers (17) categorized as atopic by positive skin test and a personal history. Atopic subjects showed a greater pulmonary response than non-atopic workers in the early stages of cotton processing. Similar results have also been reported from other environments in which workers are exposed to products derived from the cotton plant. Jones et al (18) reported that post-shift declines in FEV_1 in workers in cottonseed crushing mills were significantly related to atopic status. Atopy was defined as two or more positive skin tests to a battery of ten geographically common allergens. Although both the atopic subjects and non-atopic subjects responded to the aerosols of cotton dust the findings of this study confirm that individuals with mild atopy may be at greater risk of responding more severely to aerosols of cotton dust than the non-atopic population. With lower dust levels in US textile mills, workers that respond in these environments may include a larger proportion of atopics. The recent study by O'Neil et al (17) supports this hypothesis in that workers identified as responders in the opening, carding and spinning areas included a larger proportion of atopics. A factor that may contribute to the increased proportion of atopics among responders is the reduced selection pressure due to lower dust levels. Atopic workers that historically would have selected themselves out of dusty environments can now tolerate the less dusty environment. Another factor that may be important to the reaction at the lower levels of cotton dust, but which has not been evaluated experimentally, is that the increased airway reactivity characteristic of atopic individuals may predispose them to react in any dusty environment. The reaction of atopics to low levels of cotton dust could be a generalized response to their environment, a response to specific agents in the dust or a combination of the two.

Nasal Lavage Data

Nasal lavage was performed on a total of 57 subjects. Nine of the subjects (15%) were unable to perform lavage on the first attempt. In most cases either all or a portion of the lavage sample was swallowed prior to the completion of the 10 second holding period. For these subjects a second lavage was performed but subsequently discarded since the effect of sequential lavage on cell numbers has not been defined. As the study progressed the procedure for administering the lavage fluid and subject coaching improved and sample loss was reduced. For subjects successfully completing the lavage procedure on the first try, the mean volume recovered for the pre-exposure lavage was 6.5 ml (sem 0.2) compared to 6.4 (0.2) for post-exposure lavage. This compares to a mean

recovery of 6.8 (+/- 1.0 ml) for pre-and post-exposure lavage in subjects exposed to ozone (9,10). There was no significant difference in the pre-to post-lavage volume indicating that the exposure to cotton dust did not affect the performance of the lavage maneuver.

The range for total cell counts in nasal lavage of unexposed subjects was from <1000 to 2.9 x 10^5. The majority of subjects (77%) had pre-exposure lavage counts of 10,000 cells/ml of nasal lavage or less. In a previous study using subjects similar to those used in Sweden, 50% of the subjects had lavage counts of 10,000 cells/ml or less (9). Of the total subjects evaluated, 19% had counts of <1000 cells/ml, 58% had counts ranging from 1000 to 10,000 and 23% had counts in excess of 10,000 cells/ml. These data compare to 8%, 42% and 50% respectively in a similar cohort of volunteers in the United States (9). These differences are relatively minor and may reflect differences in the two populations evaluated or sample size.

For all subject participating in the study there was a significant increase in the number of cells recruited after exposure to cotton dust (Figure 1). The mean pre-exposure cell count increased from 1.8 x 10^4 cells/ml to 3.3 x 10^4 after exposure to cotton dust. The difference between pre-and post-exposure cell numbers was significantly different ($p<0.05$). The influx of cells in response to cotton dust is in agreement with results from other studies, using both animals and lavage of subjects, which have demonstrated that aerosols of cotton dust cause the influx of cells to the airways (3-6). While this finding in not new it does indicate that nasal lavage may be a non-invasive procedure that can be used reliably to assess the pulmonary response to aerosols of environmental or occupational pollutants. Other studies have demonstrated that exposure to oxidant gases causes a marked inflammatory cell influx in the nose in both normal (9,10) and asymptomatic allergic subjects (8). The two-fold increase in cell count observed after exposure to cotton dust is similar to the 2.5 fold increase reported by Graham for exposure of healthy non-smoking subjects to 0.5 ppm ozone for 4 h on two consecutive days (10), but less than the sevenfold increase reported by Bascom et al (8) in asymptomatic allergic subjects exposed to similar levels of ozone. These data suggest that the response, as measured by nasal lavage, is similar to that of ozone but that the response to cotton dust is less marked.

For confirmed atopics and non-atopics there was no difference in the mean pre-lavage cell counts, although the mean pre-exposure non-atopics cell count was less than (1.4 x 10^4) the pre-exposure cell count for confirmed atopics (2.7 x 10^4). This trend agrees with the observations of Halopainen (19) that subjects with atopic rhinitis had greater numbers of PMN's in nasal smears than a comparable group without symptoms. The average baseline counts seen in this study were intermediate to those reported for non-atopics (1.09 x 10^4) and asymptomatic allergic subjects (11.5 x 10^4) (8,10). This supports the differences seen in the baseline counts reported for the atopics and non-atopics. Histopathologic studies have demonstrated marked inflammation in the airways of asthmatics with infiltration of inflammatory cells, particularly eosinophils and disruption of airway epithelium. Similar, but less severe pathologic changes have been observed for subjects with asymptomatic bronchial hyperresponsiveness. These data would suggest that nasal lavage is a tool that can be used to assess the state of airway inflammation which may be an indicator of airway hyperresponsiveness.

Pre-to post-exposure comparisons of atopics and non-atopics are shown in Figure 1. Non-atopics showed a significant increase in the number of cells recruited to the nasal mucosa ($P<0.05$). Although there was a similar trend for the confirmed atopics the increase in cell number was not significant. These data suggest that non-atopics were more responsive than confirmed atopics with respect to cell recruitment to the nasal mucosa. However, the higher cell counts in the pre-exposure lavage of the confirmed atopics indicate an elevated state of inflammation and those subjects may have been less able to respond to the exposure with a measurable influx of inflammatory cells. This lack of a cellular responsiveness among atopics does not correlate with the observed changes in FEV_1. However, increased airway reactivity has been associated with the degree of airway inflammation (20) and the elevated cell counts in the pre-exposure lavage of the atopics would identify this group as being more likely to have a increased airways response.

The nasal lavage data were sorted into three categories based on pre-lavage data: <1000, 1000-10,000, and >10,000 cells/ml. For non-atopics, subjects in the first two pre-exposure categories had a significant increase in cell number after cotton dust exposure (Figure 2). Confirmed atopics did not show a significant increase in lavage cell number for any of the three categories. These data demonstrate that atopic subjects remain less responsive than non-atopics even with similar starting cell counts and would suggest other factors, in addition to cell counts, are important in the relationship between inflammation and airways reactivity.

As the pre-exposure cell count increased for non-atopics the magnitude of the change in FEV_1 after exposure was larger. The mean change in FEV_1 for each pre-exposure lavage category was -6.09%, -9.72%, and -5.26% for atopics (<1000 cells/ml, 1000-10,000, and >10,000, respectively) and -3.27%, -5.34%, and -5.42% for non-atopics. These data would suggest that increasing cell number in pre-exposure nasal lavage reflects an elevated inflammatory state and increased airway reactivity.

Summary

1. Aerosols of cotton dust caused an influx of neutrophils to the nasal mucosa similar to that reported more distal in the lung from lavage studies. This suggests that nasal lavage may be a reliable non-invasive procedure to assess the pulmonary response to environmental and occupational pollutants.

2. The pre-exposure lavage cell counts were higher in confirmed atopics than non-atopics suggesting a difference in the baseline state of inflammation for these groups.

3. Overall, non-atopics showed a significant increase in the number of cells recruited after exposure to cotton dust suggesting a greater quantitative cellular response than atopics.

4. At comparable baseline levels of cells in pre-exposure nasal lavage the quantitative cellular response of non-atopics was larger than atopics. These data suggest other factors not measured by this study contribute to airway responsiveness in atopics.

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Table 1. Descriptive characteristics of subjects exposed to aerosol of cotton dust

	AC	NA	ANC	TOTAL
N	20	32	5	57
MALE	17	19	2	38
AGE	23.2 (3.5)	24.7 (3.7)	22 (1.6)	23.8 (3.7)
FEV_1% PRED.	103 (7)	111 (8)	119 (14)	110 (9.4)
FEV_1/FVC	86 (7)	84 (7)	92 (3)	85 (7)
PHADIATOP	32.4 (19.4)	1.5 (1.7)	14.1 (13.0)	13.4 (19.0)
DUST LEVEL MG/M³	1.2 (.1)	1.1 (.2)	1.1 (0.2)	1.1 (0.2)

Parenthesis = Standerd Deviation
NA = Non-Atopics
AC = Atopics - confirmed by a physician
ANC = Atopics - not confirmed by a physician

Table 2. Mean spirometric changes of subjects exposed to aerosol of cotton dust

	OVERALL	ATOPICS	NON-ATOPICS	
FEV_1	-6.0 (0.6)	-8.3 (1.3)	-4.9 (0.7)	P<.005
FEF_{25-75}	-10.5 (1.2)	-13.4 (2.5)	-8.6 (1.5)	P<.05
FVC	-3.4 (0.5)	-5.2 (0.8)	-3.0 (1.0)	P<.02

(SEM)

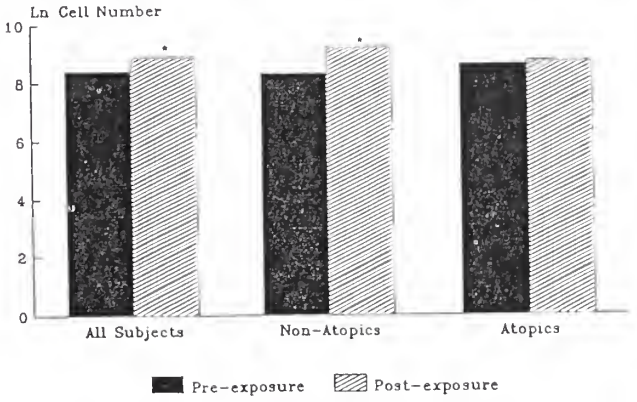

FIGURE 1. CHANGE IN CELL NUMBER IN NASAL LAVAGE OF ATOPIC AND NON-ATOPIC SUBJECTS SORTED BY PRE-LAVAGE COUNTS OF <1000, 1000-10,000 AND >10,000 CELLS/ML

*p<.05

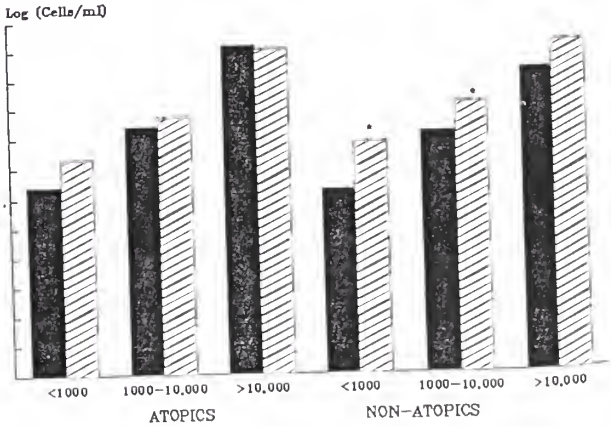

FIGURE 2. CHANGE IN CELL NUMBER IN NASAL LAVAGE FOLLOWING EXPOSURE TO COTTON DUST (LN-CELLS/ML)

*p<.05

AN EVALUATION OF AVAILABLE METHODS FOR MEASURING CONDENSED TANNINS IN AIRBORNE SAMPLES OF VERTICAL ELUTRIATED COTTON DUST

Robert R. Jacobs, Associate Professor
and
Kelly Atchley, Graduate Student
University of Alabama at Birmingham
School of Public Health, Birmingham, AL

Abstract

Condensed tannins are a heterogenous group of plant natural products suspected to play a role in the etiology of byssinosis. It is not known, however, if the level of condensed tannin is present in aerosolized dust in sufficient quantity to induce a response among workers in this industry. We sought to determine if an existing method used for measuring tannin in plant tissue, a spectrophotometric method based on the absorbance of a 1% solution of tannin ($E^{1\%}$), was sufficiently sensitive to analyze dust deposited on vertical elutriator (VE) filters. This technique was compared to tannin levels measured by standardizing with a purified tannin. There was a significant correlation between the two methods. The tannin levels based on standardization with a pure tannin were 40% higher than the $E^{1\%}$ method. The limit of detection (LOD) for the standardized technique was 1.4 μg tannin/mg dust. The LOD for the $E^{1\%}$ method was estimated to be 2.3 μg tannin/mg dust. Based on these analyses it appears that both techniques are near the limit of detection for tannin in dust contained on VE filters; however with appropriate sampling and analytical modification it should be possible to measure the levels of tannin in dust collected on vertical elutriator filters.

Introduction

A number of studies have been published which show a relationship between pulmonary inflammation caused by inhaled cotton dust and airborne levels of endotoxin (1). However, it is unlikely that endotoxin alone is responsible for the complete biological activity associated with cotton dust. The focus on endotoxin is in part because it is the only specific component of cotton dust that has been measured in airborne dust samples. Other biologically active components in the dust may covary with endotoxin. A variety of natural products and microbially derived contaminants with demonstrated biological activity *in vitro* have been isolated from cotton dust yet they have not been measured in aerosolized dust samples (2). One compound found in high quantities in bulk dust samples is vegetable tannin.

Vegetable tannins are defined as water-soluble polyphenolic compounds with molecular weights ranging from 500 to 3,000; additionally, they can be further characterized as having special properties such as the ability to precipitate alkaloids, gelatin, and other proteins (3). Tannins are generally categorized into two groups, the hydrolyzable and nonhydrolyzable tannins or proanthocyanidins (condensed tannins). Condensed tannins are further characterized as condensation products of flavan-3-ol (catechin) compounds with C_4-C_8 or C_4-C_6 interflavan bonds (4). Hydrolyzable tannins, also described as gallo- and ellagi-tannins, can be split by acids or bases into sugars or related polyols and a phenolic acid.

In analytical assays, tannins are usually described as extractable or residual. Extractable tannin is condensed tannin that is soluble in various solvents. Residual tannin is the material left behind after extraction. Intuitively, the residual tannin is of little interest biologically because only extractable tannin is thought to induce a toxic response in a biological system.

Condensed tannins are thought to be important agents in a plant's natural ability to resist insects and therefore are of interest with respect to their ability to initiate a biological response (5). Condensed tannin isolation from flower buds of cotton has been shown to retard larval growth of the tobacco budworm (5). Lane and Schuster also reported that several strains of cotton which have increased resistance to spider mites contain elevated levels of condensed tannin.

Rohrbach, using a refinement of a technique developed by Taylor *et al*, (6) prepared condensed tannin free of low molecular weight hydrolyzable tannin and tannic acid (7). In vitro assays using condensed tannin prepared by this technique have been shown to alter alveolar macrophage function (7), inhibit the electrophysiologic properties of tracheal epithelial cells (8), activate T-lymphocytes (9), induce the recruitment of polymorphonuclear neutrophils (PMNs) to the terminal airways (10), activate platelets (11), and damage endothelial cells (10). Although a wide array of biological activity has been shown using *in vitro* cell systems, the relevance of tannin in the development of a pulmonary response to cotton dust is unknown since this activity has only been characterized *in vitro* and at doses that may not be relevant for typical workplace exposures.

At present, no data exist describing the levels of tannin in airborne dust found in occupational settings and only limited analysis has been performed to estimate the concentrations of condensed tannins in bulk cotton dust samples. This study sought to determine if the theoretical absorbance method ($E^{1\%}$) as used by Bell and Stipanovic (12) for measuring tannin in plant tissue was sufficiently sensitive to measure the levels of tannin in vertically elutriated cotton dust samples and to evaluate the accuracy of the $E^{1\%}$ method relative to tannin levels measured using a purified standard control.

MATERIALS AND METHODS

The butanol-acid assay as performed by Bell and Stipanovic (12), was modified according to Porter, Hrstich, and Chan[13] and Ann E. Hagerman, Miami University, Oxford, Ohio (personal communication).

Bulk Cotton Dust: Bulk cotton dust from condenser filters was collected in 1985/86 from carded cotton of the MQ-119 E, F, and D series. The MQ-119 E, F, and D cotton was of the same variety and was grown in experimentally controlled plots located in California, Texas, and Mississippi, respectively. The bulk dust was stored in a desiccator in the dark at room temperature for one week prior to analysis. However, before the one week pre-analysis period, there was no environmental control for storage of the samples.

Standard Tannin: The condensed tannin used as a standard was prepared in 1991 by Dr. A. A. Bell(Cotton and Grain Crops Research Laboratory, College Station, Texas) using the procedure of Chan et al (16). The purified condensed tannin was received as a 95% pure lyophilized preparation. The purity of the product was confirmed using HPLC (Personal Communication -Dr. Al Bell). The purified condensed tannin was stored in the dark at room temperature under desiccation prior to analysis.

Cotton dust extraction: Twenty-one 10 mg samples of bulk cotton dust from each location were assayed using the modified butanol-HCl procedure. The sample size of 10 mg was selected based on preliminary data defining the limit of detection (LOD) of the method using the standard tannin. Each 10 mg aliquot of dust was placed in a sterile screw cap culture tube and extracted with 10.0 ml of 50:50 acetone:water reagent. Each sample was extracted for one hour at room temperature in a Glas-Col® Apparatus Company rotating mixer. After extraction, each tube was centrifuged for five minutes at 3,000 RPM and one ml of the supernatant removed for butanol-acid analysis.

Six ml of 95:5 butanol-HCl reagent and 0.2 ml of 2% ferric ammonium sulfate were added to one ml of the acetone:water extract and vortexed. The sample was loosely capped and placed in a dry bath heating block for 50 minutes at 98°C. After the tube had cooled, an

aliquot was transferred to a disposable polystyrene cuvette with a 1 cm path length and the absorbance read at 550 nm. The absorbance of a blank containing only sample solvent, butanol acid, and iron served as the reference sample. When using the $E^{1\%}$ tannin calculation method, the maximum absorbance that occurred between 540 nm and 560 nm was determined.

Dust calibration curves were developed for each of the three bulk dust samples. Each bulk dust calibration curve was used to determine the actual $E^{1\%}$ value so percent tannin could be calculated using the $E^{1\%}$ method. The $E^{1\%}$ value for each of the three dusts was 250, indicating no difference between the three growing locations. The data generated from these studies were used to determine the relationship between the $E^{1\%}$ method and the concentration measured from a calibration curve based on purified tannin.

Standard calibration curves were prepared before and after each group of 21-ten milligram dust samples. Dilutions of the purified tannin were prepared as follows. Two mg of purified tannin was suspended in 2 ml of the butanol-acid/iron reagent. Serial dilutions were prepared in the butanol-acid/iron reagent to achieve the following concentrations (mg/ml): 0.01, 0.0157, 0.02, 0.0313, and 0.0625. The correlation coefficient for each of the generated curves was $R > 0.99$. The average slope and intercept was used to formulate the following expression:

Y (absorbance) = 15.97134 X (mg tannin) + (-0.0104)

This equation was used to determine the tannin concentration in the dust samples and for comparison with the $E^{1\%}$ method. The method of Long and Winefordner was used for the calculation of the limit of detection (LOD) using the data from the standard tannin calibration curve (14).

Statistical Analysis

The relationship between the $E^{1\%}$ calculation and the standard tannin calibration method was evaluated by linear regression. The values obtained from the standard tannin calibration method were considered the independent variable for the regression analysis because we postulated that they would provide the best estimate of the amount of tannin in a given sample. A one-way ANOVA was used to compare the amount of condensed tannin in bulk dust samples from cotton grown in different geographical locations (California, Mississippi, and Texas). All statistical tests and procedures were performed using Strategy Plus Incorporated's EXECUSTAT statistical software, version 2.1. Differences were considered significant at $p \leq 0.05$.

Results and Discussion

The primary objective in this investigation was to determine if an existing technique developed by plant pathologists to assay the amount of tannin contained in plant tissues could be used to estimate the amount of tannin in aerosolized cotton dust from VE filters. Samples of bulk cotton dust from a single variety of cotton grown in three different growing locations were evaluated for tannin content using both the $E^{1\%}$ procedure and standard curves prepared from purified tannin to estimate tannin concentration. The levels of tannin measured by each technique for the three dust samples are shown in Table 1. The level of tannins in the dust from the California grown cotton, regardless of method, was significantly higher than either the dust from Texas or Mississippi ($P<0.05$). There was no difference in tannin levels for the Mississippi and Texas dust.

These data confirm previous studies showing that dust from cotton grown in California has higher levels of tannin than cotton grown in either Texas or Mississippi (15). Since these dusts were from the same variety of cotton the differences in tannin level likely reflect differences in growing conditions, cultivation practice, and harvest. For example, there is more "green" tissue in California grown cotton because the plants are not killed either by a freeze/frost or by chemical application prior to harvest. Numerous studies have shown that as the plant scenesence the level of tannin decreases.

The theoretical absorbance ($E^{1\%}$) determined from standard curves prepared from each of the three bulk dusts was 250. This is similar to the $E^{1\%}$ of 240 used by Bell and Stipanovic (12) and 270 by Lane and Scheuster (5). We had anticipated that the $E^{1\%}$ values would be different for the different growing locations because the value reflects the ratio of delphinidin and cyanidin. However, the common value may reflect the fact that dust from a single variety of cotton was used for the analysis and that growing area does not influence the ratio of delphinidin to cyanidin. Other variety:area dust combinations should be evaluated to determine the variability in the $E^{1\%}$ values.

The levels of tannin for each of the three dusts are lower than those reported in the literature. Bell reported than bulk samples of a 1984 milled Mississippi dust contained 1.7-3.8 % condensed tannin, while 1982 and 1983 milled Texas dusts contained 4.6-7.3 and 2.6-5.2 % condensed tannin, respectively (15, 12). The levels of tannin in the Texas and Mississippi dusts in this study were both less than 0.15% using the $E^{1\%}$ technique (Table 1). While these differences may reflect plant variety, several other factors may account for lower levels observed in this study, including: the age of the bulk dust analyzed and the lack of environmental control after sample collection. Environmental control is important in order to prevent microbial growth or other factors which may reduce the amount of tannin. The age of a sample is intuitively important in that the sooner a sample is analyzed the less chance that degradation will occur either from microbial enzymes or from oxidation due to sunlight exposure. To stabilize a sample for analysis, it should be kept in a cool dark place under desiccation immediately after collection and analyzed as soon as possible.

The differences in tannin levels may also reflect the nature of the sample. The bulk dusts analyzed in this study were from condenser waste collected from an air handling unit and represent a size-selected aerosolized dust. A previous study has reported that the concentration of tannin in the smaller fractions of a sieved dust were less than in the larger fractions (12). Therefore tannins may represent a smaller fraction of the total material contained in an aerosolized dust.

The relationship between the $E^{1\%}$ calculation and the purified tannin standardized assay is shown in Figure 1. A statistically significant relationship was observed between the tannin levels estimated by the $E^{1\%}$ method and values determined from the purified tannin calibration curve ($p<0.05$). The following relationship was defined:

% Tannin = -0.289 + 0.622 (% tannin Standard)

Based on evaluation of the purified tannin standard curves the limit of detection (LOD) for the standardized tannin method was 1.4 μg tannin/mg dust. An LOD was not determined for the $E^{1\%}$ calculation, but based on the relationship between the two methods the LOD for the $E^{1\%}$ calculation is approximately 2.3 μg tannin/mg dust.

Using the LOD values and the tannin levels from Table 1 we can determine if the sensitivity of either method is adequate to measure tannin in VE filter dust samples for this cotton dust (Table 2). The data in Table 2 are based only on the data from the Texas samples. Similar tables could be constructed for the California and Mississippi samples. These data suggest that for the Texas and Mississippi dusts only the method using the standard tannin would be sufficiently sensitive, without modification, to measure the tannin levels on VE filters. Both methods would appear to be adequate for evaluating the tannin levels in California VE dust samples, although dust levels of > 0.25 mg will be required for the $E^{1\%}$ tannin analysis. Use of the $E^{1\%}$ method for routine analysis of tannin on VE filters will require that larger quantities of dust be collected. This may be achieved by longer sampling times, however quality control considerations may limit the use of this strategy for sample collection. Alternatives that may be satisfactory include: pooling VE filters; using high volume sampling pumps and filters; or omitting the acetone-water extraction and determine total tannins by direct analysis of the sample with the butanol acid reagent. This latter approach would increase sensitivity of analysis, however, the results may not represent biologically active (extractable) tannin.

The butanol acid assay is selective for condensed tannins (proanthocyanidins). This assay selectively cleaves the flavonoid subunits via oxidation to produce cyanidin and delphinidin (anthocyanidins) pigments. Although this method is frequently used to measure tannins it is but one of several published techniques and is not problem-free. Catechin, a component molecule of tannin, reacts with the butanol acid reagent to produce a yellow pigment which gives a positive absorbance value that will overestimate the amount of condensed tannin. Furthermore, leucoanthocyanidins, which are flavan-3,4-diol monomers, can react with the butanol acid reagent to form anthocyanidin pigments; however, they will generally decay before absorbance is measured due to their instability.

An additional limitation of the butanol acid assay is due to delphinidin (Λ_{max}=558nm) having a greater absorption maximum than cyanidin (Λ_{max}=547nm). The ratio of cyanidin and delphinidin in a particular sample may affect the estimation of the amount of condensed tannin present. Given an extract containing a large amount of delphinidin, one may overestimate the amount of condensed tannin due to the different absorbancies (17). This error may be compounded by what appears to be an incorrect assumption in the derivation of the $E^{1\%}$ method. The underlying assumption of the procedure is that the absorptivity coefficients of cyanidin and delphinidin (εcy=150, εdl=300) are equivalent. However, one will find that the above coefficients are not equal and the use of this method to determine tannin concentration may result in an over or under estimation.

In conclusion, the $E^{1\%}$ method for measuring tannin is relatively simple and does not require a purified standard. However, the limitations of the technique should be acknowledged when reporting for different types of tissue. The method as used in this study does not appear to be adequate to measure the levels of tannin in dust from VE filters without modification. Consideration should be given to developing a standard tannin preparation that could be used by different laboratories to calibrate the butanol acid condensed tannin assay.

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Table 1. Differences in tannin levels in bulk dust from the same variety of cotton grown in different locations.

GROWING LOCATION (n)	% TANNIN MEAN (SD)	Std Curve MEAN (SD)
California (21)	0.73 (0.11)*	1.6 (0.17)*
Mississippi (21)	0.13 (0.04)	0.68 (0.07)
Texas (21)	0.14 (0.006)	0.68 (0.09)

* Significantly different P=0.05.

Table 2. Theoretical levels of tannin in Texas cotton dust at levels normally found on VE filters.

Dust Level (mg)	Theoretical Absorbance[a] Level (μg)	Detectable (YES/NO)	Tannin Standard[b] Level (μg)	Detectable (YES/NO)
0.25	0.35	NO	1.7	YES
0.5	0.7	NO	3.4	YES
0.75	1.1	NO	5.1	YES
1.0	1.4	NO	6.8	YES

[a] Based on LOD of 2.3 μg; For California dust tannin on VE filters with dust levels >0.25 mg would be detectable.
[b] Based on LOD of 1.4 μg

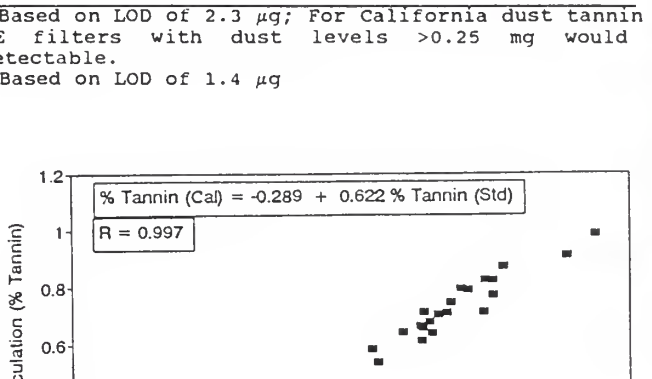

Figure 1. Calculated percent tannin vs. percent tannin from standard curve.

EVALUATION OF THE ACUTE RESPONSE AEROSOLS OF DUST FROM BATCH KIER WASHED COTTON

Robert R. Jacobs, Associate Professor,
University of Alabama at Birmingham,
Birmingham, AL,
Brian Boehlecke, Associate Professor,
University of North Carolina,
USDA-ARS, Chapel Hill, NC
Henry Perkins Jr. and David T.W. Chun
Research Chemist and Microbiologist, respectively
Cotton Quality Research Station,
USDA-ARS, Clemson, SC

Abstract

Cottons of Grade 52, "low middling light spotted or better", washed on a continuous batt system at a minimum temperature of 60°C and a water to fiber ratio of 40:1 are exempt from most of the provisions of the cotton dust standard. Batch washed cottons remain subject to all provisions of the standard. Recent studies have demonstrated that physical, chemical, and biological characteristics of cotton washed on modern batch washing systems are similar to those of cottons washed on continuous batt or rayon systems. These studies suggest that batch-kier washing of cotton is as effective as washing cotton on approved systems. In order to evaluate the biological response to dust from cotton washed on a modern batch kier system a panel of healthy subjects (n=37) was exposed to aerosols of carded dust from rayon at 0.5 mg/m^3, unwashed California and Texas cotton at 1 mg/m^3, and washed Texas cotton at 0.5 and 1 mg/m^3 in a model cardroom. Pre and post exposure spirometry was done on all subjects. There was no difference in the subjects response on any of the spirometric parameters measured for exposure to rayon, the unwashed California cotton, and the washed Texas cotton at either 0.5 or 1 mg/m^3. Exposure to the unwashed Texas cotton caused an acute pulmonary response that was significantly different than any of the other exposures ($p<0.001$). These studies indicate that batch-kier washing of cotton using modern procedures and equipment effectively removes the acute pulmonary toxicity demonstrable by spirometry at exposure levels of 1.0 and 0.5 mg/m^3.

Introduction

Cottons classified as "low middling light spotted or better" (Grade 52) and washed on a continuous batt or rayon washing system at 60°C and a 40:1 water to fiber ratio are exempt from all provisions of the Cotton Dust Standard except that medical surveillance be done every 2 years (1). In studies using human volunteers the acute potency of aerosols of dust from cottons washed on these two systems, as measured by an acute over exposure change in FEV_1, was not significantly different from no exposure (2). However, neither of these systems are widely used for wet processing of cotton, therefore the application of these washing conditions for regulatory relief is limited.

The most common system commercially available for wet processing of cotton in the United States is the batch-kier system. Batch-kier systems consist of a large vessel (a kier) that holds cotton from several bales. The cotton is packed in the kier and water at specified conditions recirculated through the cotton for several cycles. After completion of the wash cycle, the fiber is rinsed, removed from the kier, centrifuged, dried and baled.

Previous studies evaluating the human responsiveness to cottons washed on batch-kier systems were done on older commercial systems. Dust generated from these cottons caused an acute over exposure decrease in FEV_1 that was significantly different from no exposure(2). The system used for these studies required that the cotton be placed in the kier by hand after which the kier was sealed and water at specified conditions recirculated from the top to the bottom of the kier(3). Due to the hand packing of the fiber, it is likely that the fiber was unevenly wetted which would allow channeling of the wash water through the cake of fiber and cause uneven or inadequate washing.

State of the art batch-kier systems rely on mechanical packing of cotton that has been prewetted with a surfactant (4). The cotton is packed to a uniform density and then the wash water recirculated inside-out for the time specified in the washing protocol. The remainder of the washing procedure is similar to that used in older batch-kier systems. Channeling of the wash water does not occur in these systems; therefore a more uniform wash is achieved.

It is likely that washing on modern batch kier systems can remove the biological activity from cotton as effectively as either the rayon or continuous batt systems. If this can be demonstrated in studies evaluating the responsiveness of human panels to dust aerosols as well as by the measured chemical and biological attributes of the washed fiber itself, then cottons washed on modern batch kier systems should qualify for exemption from the Cotton Dust Standard. This study evaluated the spirometric response of healthy volunteers to two levels of dust of a Texas cotton washed on a modern batch-kier system. The response of the panel to washed cotton was compared to the responses to a control exposure to rayon, to the unwashed Texas cotton, and to an unwashed California cotton.

Materials and Methods

Subject Recruitment and Selection

Lifetime non-smokers without a history of asthma or previous exposure to cotton dust were recruited by radio and newspaper advertisement. Subjects were initially screened by telephone and if qualified, invited to a follow-up screening session at the laboratory. Laboratory screening consisted of spirometry, administration of a more detailed medical and occupational questionnaire, and if not excluded by questionnaire response, collection of a sample of venous blood for evaluation of atopic status. The questionnaire asked about personal and family history of allergy and previous exposure in dusty environments. Women of child bearing age were asked to complete a pregnancy test. Criteria for exclusion from the study included an FEV_1 or FVC <80% predicted; FEV_1/FVC ratio <70%; positive pregnancy test; previous history of exposure to cotton dust; or chronic respiratory illness.

A multiple Rast test for IgE antibodies to common aeroallergens (Phadiatop®, Cap system, Pharmacia Diagnostics AB, Uppsala, Sweden) was used to confirm atopic status. Results of the Phadiatop are reported as positive or negative relative to laboratory determined cut off value.

Experimental Design and Exposure Sequence

Subjects selected for the study were asked to participate two consecutive days a week for five weeks. Each week participants reported the day prior to dust exposure for methacholine bronchoprovocation testing. The next day, prior to entering the cardroom, participants completed baseline spirometry. Each participant remained in the cardroom for 5 hours during which lunch was provided; restroom breaks were allowed as needed. Subjects were removed from the cardroom after 5 hours and spirometry and methacholine testing performed. A symptom questionnaire was completed immediately post exposure and again at 24 hours. The same pre test and exposure day was maintained for each subject during the five week study. Only data pertaining to the spirometric response to aerosols of card-generated cotton are given in this presentation.

On initial screening, subjects had been asked if they had hayfever confirmed by a physician. Those responding

affirmatively were categorized as atopic for selection purposes. By this criteria approximately one-half of those selected were atopic. Atopic and non-atopic participants were randomly assigned to four equal sized groups. During the first four weeks each group was exposed each week to one of the four exposure conditions described below in a Latin squares design. This resulted in all participants being exposed to each condition by the end of the study. During the fifth week all subjects were exposed on their specific day to aerosols of the Texas washed cotton at 0.5 mg/m³. Atopic status as determined by the Phadiatop was available later and is used in the analysis.

Study Cottons

Four exposure conditions were evaluated during the first four weeks of the study. Dust was generated from four types of fiber: Rayon, California and Texas unwashed cottons and the Texas cotton after washing. The rayon was 1.5 denier, 1.5 inches, bright, standard textile grade from BASF. The California cotton was a blend of six bales of Grade 31 (middling). The Texas cotton was a blend of 34 bales of predominantly Grade 52 (low middling light spotted).

The target dust level for each cotton was 1 mg/m³. The target dust level for rayon was 0.5 mg/m³ which was the maximum achievable for the carding conditions and minimum room ventilation criteria.

Exposure Room and Environmental Monitoring

All exposures took place in an experimental cardroom at the USDA-ARS Cotton Quality Research lab in Clemson, South Carolina. The dimensions of the cardroom have been described previously (8). Chairs for subjects were situated around the perimeter of the cardroom and subjects were asked to move to a different location at hourly intervals. Dust levels were controlled by adjustment and balance of card production rates and air supply and exhaust rates (9). Dust levels were monitored using four vertical elutriators (VE) at different positions in the cardroom and with a portable continuous aerosol monitor (PPM Inc, Knoxville, TN). The particle size distribution was monitored using an Anderson Series 210 Cascade Impactor. All cottons and rayon were processed through blender-feeders and three stages of blending, opening, and cleaning on a finisher picker to prepare laps for feeding a Saco Lowell carding machine. Carding was at 30 lbs/hr for the cotton and 60 lbs/hr for the rayon.

A separate carding evaluation was conducted to determine the relative dust levels of washed and unwashed Texas cottons at standard carding conditions. For this evaluation carding speed was 45 lbs/hr and 750 cfm of air was used on the local capture cleaning equipment. Temperature was maintained at 75°F ± 2°F and relative humidity at 55% ± 2% for all carding runs.

Ambient air was sampled for total viable airborne bacteria with six-stage Anderson air samplers (Anderson 2000 Inc., Atlanta, Georgia) operated at 0.028 m³/min for 15, 30, 60, or 120 seconds. The samplers were loaded with 90 mm plastic petri plates filled with 40 ml of trypticase soy agar [g trypticase soy broth, 20 g agar and 5 g yeast extract per liter and cycloheximide (50 µg/ml)]. The plates were incubated for 22-30 hours at room temperature (RT;24.1±03°C).

For airborne endotoxin, VE filters were extracted in 50 ml pyrogen free water (PFW) for 1 hour in pyrogen free borosilicate glass containers. Appropriate serial 10-fold dilution were evaluated by the kinetic chromogenic modification of the Limulus amebocyte lysate (LAL) assay (BioWhittaker).

Washing Conditions

The following conditions were used for a low-temperature, high water-to-fiber ratio wash of the blended Texas cotton.

1. Blended cotton was processed through typical textile mechanical opening and cleaning machines to produce small fiber tufts for pre-wetting.
2. The opened cotton was fed to a "cakemaker" and pre-wetted with a surfactant (0.04% Penn B, on weight of solution).
3. Three doughnut shaped cakes of cotton were place in each kier for washing.
4. The kier was filled with 1100 gallons of water at room temperature containing 12 pounds of surfactant. The water temperature was elevated to 60°C, circulated for 3 minutes, and drained to the sewer.
5. Step 4 was repeated 6 times.
6. The kier was filled with 1100 gallons of water without surfactant at room temperature. The water temperature was elevated to 60°C, circulated for 3 minutes, and drained to the sewer.
7. Repeat step 6.
8. Apply vacuum to kier to help remove excess solution.
9. Remove cakes individually and centrifuge to about 50% moisture content.
10. Dry in tunnel dryer at 125°C - 145°C to target moisture content range of 4% - 7%.
11. Feed through a mechanical opener to the bale press.

Total water-to-fiber ratio, including that used in cakemaking, was approximately 40:1. Both the wash solution and the rinse water were pumped from the inside to the outside of the cakes of cotton in the kier. During recirculation the solution was pumped in a closed loop system through the cotton into an overflow tank and back through the cotton. Both the wash solution and rinse water were dumped to the sewer and not reused. The total kier cycle time was about 1¼ hours.

Data Analysis

To adjust for differences in age, height, and sex, baseline spirometric data were converted to percent predicted using Knudson and co-workers equations (10) adjusted for race. All four groups were combined to determine the response to each type of exposure. Data were analyzed using the General Linear Model procedure for analysis of variance and least squares adjustment of means for missing data (SAS, Cary, NC). Differences were considered significant at the P≤ 0.05 level. For viable microbial counts the data were converted to logs and evaluated by GLM ANOVA. Multiple comparison was done using Duncan's range test.

RESULTS

Study Population

The characteristics of the study participants are shown in Table 1. Of the 38 participants, 79% were female with the average age of 33 years old. Fifteen subjects were categorized as atopic by the reference laboratory's criterion on the Phadiatop assay for IgE antibodies to specific common aeroallergens. A larger portion of the non-atopic subjects were female (87 vs 67%) and non-atopics were on average 3.5 years older than atopics. Although atopics had slightly higher baseline values for FEV_1 and FVC as percent of predicted than the non-atopic subjects, these differences were not statistically significant.

Eighteen of 38 subjects gave a personal history of atopy confirmed by a physician on questionnaire. Only eight of the these subjects (44%) were classified as atopic by the Phadiatop (Figure 1). Of the subjects classified as atopic by the Phadiatop 30% gave an negative history of atopy.

Exposure Conditions

The mean VE dust concentrations for the four exposure days for each condition, are shown in Table 2. The dust level for rayon was approximately one-half the level for the cotton exposures except for week five. The rayon

concentration ranged from 0.41 to 0.50 mg/m³ with 64% of the particles less than 4.4 μm in aerodynamic diameter. For California, Texas unwashed, and the Texas washed cotton the dust levels were maintained at approximately 1 mg/m³. During the fifth week all subjects were exposed to washed cotton at 0.5 mg/m³. For each cotton exposure the range over the four days was less than ± 8% of the individual means. For California cotton 61% of the particles had a mean aerodynamic diameter <4.4 μm and for Texas unwashed cotton 73% of the particles were <4.4 μm. For the Texas washed at both 1 and 0.5 mg/m³ only 58% of the particles were <4.4 μm indicating a shift towards larger particles for the washed cotton.

Airborne total bacterial counts were highest for Texas unwashed and lowest for rayon (Table 2). There was no significant difference between the levels of airborne bacteria for the rayon and California exposures. Airborne bacteria levels generated from the Texas washed cotton at 1 mg/m³ were significantly lower than for the unwashed cotton ($P < 0.05$). Reduction of the dust level of washed cotton to 0.5 mg/m³ reduced the airborne bacterial levels by approximately 50%. The airborne bacterial levels in dust generated from washed cotton at both 1 and 0.5 mg/m³ were significantly higher that either the California or rayon exposures ($p < 0.05$). The bacterial levels for both California and rayon were similar to the levels measured in the cardroom with no fiber being processed at normal exposure room ventilation conditions. Statistical comparison between control room levels and the different exposures could not be done because control room conditions were measured on only one day. The control room levels are similar to those reported in previous studies in this chamber (5).

Lint and airborne endotoxin levels are shown in Table 3. For the lint, rayon had the lowest value (2.3 Eu/mg) followed by Texas washed (72 EU/mg), California (82 Eu/mg) and Texas unwashed (260 Eu/mg). The magnitude of the differences in the endotoxin on the lint was reflected in the airborne levels of endotoxin. There was no significant difference in the levels of airborne endotoxin between the rayon exposure, the California exposure or the Texas washed exposure at 1.0 mg/m³. Airborne endotoxin for Texas washed at 0.5 mg/m³ were not measured. The level of airborne endotoxin associated with the unwashed Texas cotton was significantly higher than any of the other exposures.

Spirometry

There was a decline in both the FEV_1 and the FVC after all exposures (Table 4). However, the changes were significantly different from zero only for the Texas unwashed cotton and the Texas washed cotton at 1 mg/m³. Only the Texas unwashed resulted in a significant decline in FEV_1/FVC ratio. The group mean changes for rayon, California cotton, and the Texas washed at 0.5 mg m⁻³ were not significantly different from zero for any of the spirometric parameters measured.

Mean pre to post exposure change in spirometric parameters as percent of pre-exposure values are shown in Table 5. There was no difference among the responses to dust from rayon, California cotton and Texas washed cotton at 0.5 or 1 mg/m³ for any of the spirometric parameters measured. For unwashed Texas cotton the mean change in FEV_1, FVC, and FEV_1/FVC was significantly larger than for all other exposure conditions ($P<0.0001$).

The FEV_1 response of subjects stratified by atopy is shown in Figure 1. There was no significant difference between atopics and non-atopics at any of the five exposure conditions. However, the drop in FEV_1 of atopics was larger than that for non-atopics for all exposures except California cotton.

For unwashed Texas cotton the group mean change for FEV_1 was significantly different from zero for both atopics and non-atopics even when using a conservative p-value for this post-hoc test (<0.001) (Table 6). A significant difference was seen only in atopics for FVC. For washed cotton at 1 mg/m³ the mean change in FEV_1 was significantly different from zero for atopics only ($p < 0.001$).

Discussion

Previous studies have shown that the acute airway reactivity of cottons is not completely removed by washing on older batch kier systems. Table 7 summarizes the previous studies evaluating the response of human panels to cotton washed at 60°C and a 40:1 water to fiber ratio on the older batch-kier system and the OSHA approved continuous batt system. The data are expressed as a dose-response slope for the acute change in FEV_1 at a projected exposure of 1 mg/m³. For unwashed cotton the slope of the dose response curve was significantly different from zero. The slope of the response to the same cotton washed on a continuous batt system was not significantly different from zero. Exposure to cottons washed on the batch-kier system resulted in a significant airway response for all trials.

Results from this study show that the acute airway potency of cottons washed on a modern batch kier system is no different than a control exposure to Rayon at 0.5 mg/m³. The group mean decline for washed cotton at 1 mg/m³ was -2.4% and -0.88% at 0.5 mg/m³. These group mean values are less than the change of -2.7% projected at 1 mg/m³ for a similar cotton washed on the approved continuous batt system. Furthermore, at 0.5 mg/m³ the group mean response was not significantly different from zero for any of the parameters measured.

Subjects used in the study evaluating the continuous batt system had been selected for pulmonary responsiveness during screening. It is likely that these subjects represented a sensitive population, therefore perhaps a more appropriate comparison would be the response of the atopics. The group mean response for ΔFEV_1 of atopics in this study to washed cotton was -3.0% at 1 mg/m³ and -1.1 at 0.5 mg/m³. The response at 0.5 mg/m³ was not significantly different from zero. These data would suggest that washing on modern batch-kier systems removes the acute pulmonary toxicity as effectively as the continuous batt system.

The effect of washing on gram negative bacterial and endotoxin on lint as published in previous studies are shown in Table 8. For the continuous batt, rayon, and older batch kier systems washing reduced the number of viable organisms on lint by 1-3 logs and the levels of endotoxin were reduced approximately one log (7). Although no data are available for the lint from this study, the magnitude of reduction in lint endotoxin by washing is similar to that observed in studies on approved systems.

Airborne endotoxin and bacterial data are available only from cottons washed on the continuous batt system (Table 9). In those studies washing reduced the levels of GNB up to two logs and endotoxin approximately one log. In this study smaller reductions were observed in total airborne bacteria, however, the reductions in airborne endotoxin were similar to those observed for cottons washed on the continuous batt system.

Another variable to consider when evaluating the risk for acute pulmonary toxicity from washed cotton is the potential for dust generation. In a previous study, washing reduced the level of aerosolized dust from the fiber by approximately 50-60% (6). In this study, at constant carding rate (45 lb/hr) and room conditions, the dust generated from Texas unwashed and washed cottons were 0.78 and 0.39 mg/m³ respectively. This represents a 50% reduction in the level of generated dust from washed cotton. Therefore the levels of dust generated from washed cottons will be reduced and it is unlikely that the higher dust level of 1 mg/m³ used in this study will routinely occur if washed cotton is used commercially. The reduction in dust levels coupled with a shift towards the larger particle sizes that occur for washed cotton would suggest that the risk for pulmonary toxicity from washed cottons is substantially reduced.

In conclusion, these data would suggest that modern batch-kier systems can effectively remove the acute pulmonary toxicity of cottons washed at 60°C and a 40:1 water to fiber ratio. The bacterial and endotoxin levels are similar to those observed in studies using the continuous batt system. The acute respiratory response was no different than an exposure to rayon and California dust. Furthermore, the magnitude of the

response observed to cottons washed on a modern batch-kier system is similar to the response observed for subjects selected for reactivity to a similar cotton washed on the continuous batt system.

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TABLE 1. DESCRIPTIVE CHARACTERISTICS OF THE STUDY POPULATION

	ATOPICS*	NON-ATOPICS*	TOTAL
N	15	23	38
MALE %	33.3	13.0	21.0
RACE (% white)	87	83	84
AGE (sd)	31.0 (8.3)	34.5 (12.0)	33.1 (10.8)
FEV_1% Pred. (sem)	106.2 (12.3)	101.8 (9.9)	103.6 (11.0)
FVC% Pred. (sem)	106.8 (13.2)	101.6 (10.4)	103.6 (11.7)
FEV_1/FVC (%) (sem)	82.5 (1.8)	83.4 (3.1)	83.0 (2.7)

* by Phadiatop

TABLE 2. MEAN AIRBORNE DUST LEVELS, TOTAL BACTERIA, AND ENDOTOXIN FOR EACH EXPOSURE CONDITION

	DUST LEVEL[1]	Log Total Bacteria
EXPOSURE	mg/m^3 (sem)	CFU/M^3 (sem)
RAYON	0.46 (0.02)	2.93 (0.013)[A]
CALIFORNIA	1.05 (0.03)	3.15 (0.086)[A]
TEXAS UNWASHED	1.02 (0.02)	5.07 (0.084)[B]
TEXAS WASHED (1.0)	1.00 (0.02)	4.52 (0.045)[C]
TEXAS WASHED WEEK FIVE(0.5)	0.49 (0.02)	4.14 (0.044)[D]
CLEAN ROOM	ND	3.16

[1] Mean of four exposure days
Means with different letters indicate significant differences;
Duncan's multiple comparison (P= 0.05)

TABLE 3. MEAN ENDOTOXIN LEVELS FOR DIFFERENT EXPOSURES

	Lint	Airborne Dust	
EXPOSURE	Eu/mg	Eu/mg	Eu/m^3
RAYON	2.27	454.9 (86.7)[a]	209
CALIFORNIA	81.93	775.5 (113.5)[a]	814
TEXAS UNWASHED	259.62	5735 (824)[b]	5850
TEXAS WASHED (1.0)	72.37	478.3 (54.4)[a]	478

Means with different letters indicate significant differences; Duncan's multiple comparison (P= 0.05)

TABLE 4. MAGNITUDE OF CHANGE IN SPIROMETRIC PARAMETERS BEFORE AND AFTER EXPOSURE TO DIFFERENT COTTONS

EXPOSURE	CHANGE IN FEV_1 (L)	CHANGE IN FVC (L)
RAYON	-0.04	-0.04
TEXAS UNWASHED	-0.15*	-0.11*
TEXAS WASHED (1.0)	-0.07*	-0.05*
CALIFORNIA	-0.04	-0.03
TEXAS WASHED WEEK FIVE (0.5)	-0.04	-0.04

* LSMEAN significantly different from zero.

TABLE 5. PERCENT CHANGE IN SELECTED SPIROMETRIC PARAMETERS AFTER EXPOSURE TO DIFFERENT COTTONS

Exposure	FEV_1	FVC
RAYON	-1.03	-0.1
TEXAS UNWASHED	-4.7*	-2.9*
TEXAS WASHED (1.0)	-2.4	-1.4
CALIFORNIA	-1.19	-0.6
TEXAS WASHED WEEK FIVE (0.5)	-0.88	-0.8

* Value significantly different from other exposures ($p<0.05$)
Model: Effect of Exposure Significant ($p = 0.0001$)

Dust

TABLE 6. PERCENT CHANGE IN SPIROMETRIC VARIABLES AFTER EXPOSURE IN SUBJECTS STRATIFIED BY ATOPY

Exposure	FEV_1		FVC	
	Non-Atopics	Atopics	Non-Atopics	Atopics
RAYON	-0.4	-1.8	-0.3	-1.8
TEXAS UNWASHED	-4.0*	-5.3*	-2.9	-3.0*
TEXAS WASHED (1.0)	-2.0	-3.0*	-0.8	-2.0
CALIFORNIA	-2.0	-0.5	-0.4	-0.8
TEXAS WASHED WEEK FIVE (0.5)	-0.7	-1.1	-0.6	-1.2

* Mean significantly different from 0 at P<0.001

TABLE 7. PREVIOUB STUDIEB EVALUATING PULMONARY ACTIVITY OF COTTONS WASHED AT 60°C AND A WATER TO FIBER RATIO OF 40:1 ON BATCH-KIER AND CONTINUOUS BATT SYSTEMS[A].

TREATMENT[B]	MQ Number[C]	DUST LEVEL	DOSE-RESPONBE SLOPE	SLOPE = O
Unwashed	101A	0.41 - 0.53	-9.4	<0.0005
Continuous Batt	101C	0.37 - 0.40	-2.7	NB
Batch-Kier	101I	0.52 - 0.53	-5.0	<0.005
Batch-Kier	91F	0.30 - 0.46	-6.5	<0.0005
Batch-Kier	95 E,F,I,K,L	0.48 - 0.51	-2.4	<0.01

[A] Data From Castellan (2)
[B] All Cotton DPL-61 Mississippi SLM Grade 61 - USDA Designate MQ-80
[C] USDA Btudy Number (See Washed Cotton Monograph)

TABLE 8. PREVIOUS STUDIEB EVALUATING THE BACTERIAL AND ENDOTOXIN ON LINT FROM COTTONS WASHED AT 60°C AND A WATER TO FIBER RATIO OF 40:1 ON BATCH-KIER, CONTINUOUS BATT SYSTEM, AND A COMMERCIAL RAYON BYBTEM[A].

EIPOSURE	MQ-NUMBER	GNB[B]		ENDOTOXIN[C]	
		UNWASHED	WASHED	UNWASHED	WASHED
BATCH-KIER	91 F	5.0	<1.9	80-400	40
	95 E,F,I,K,L	4.6	1.5	800	40
	101 I	4.9	1.6	4600	420
CONTINUOUB BATT	101 C	4.9	<1.6-2.7	833	42
RAYON	79 C	4.7	3.58	ND	ND
	79 E	6.2	3.6		
	79 G	5.1	4.1		

[A] Data from Olenchock et al. (7)
[B] Log CFU/gm
[C] ng/gm

TABLE 9. AIRBORNE BACTERIAL AND ENDOTOXIN FROM COTTONS WASHED ON THE CONTINUOUS BATT SYSTEM[A]

Treatment	GNB	Endotoxin	
	Log CFU/m^3	ng/mg	ng/m^3
MQ-111A[B]	3.68	133.2	62.6
MQ-111D[C]	1.68	5.0	1.7
Clean Room	1.61	ND	ND

[A] Data from Olenchock et al. (7)
[B] MQ-80 Cotton
[C] Washed on the Continuous Batt System at 93°C



THE EVALUATION OF ENDOTOXIN AND GLUCAN ACTIVITY OF AN ORGANIC DUST USING GLUCAN SENSITIVE AND INSENSITIVE ENDOTOXIN ASSAYS

Robert R. Jacobs and Kathryn C. Thomas
Department of Environmental Health Sciences
School of Public Health
University of Alabama at Birmingham
Birmingham, AL

ABSTRACT

Some commercially available endotoxin assays are sensitive to both endotoxin and glucan, therefore, use of these assays will not distinguish between the Limulus amebocyte lysate (LAL) activity caused by endotoxin and that of glucan. In order to assess the contribution of glucan to the LAL activity of an organic dust, samples of lipopolysaccharide (LPS), LAL Reactive Material (LAL-RM), purified β-1,3-D-glucan, and cotton dust were evaluated using a kinetic chromogenic (ACC_c) and turbidimetric (ACC_t) glucan sensitive LAL from Associates of Cape Cod, Woods Hole, Massachusetts and a commercially available LAL that is insensitive to glucan (BW-Biowhittaker, Walkersville, Maryland). For LPS there was no measurable LAL response in NaOH treated samples using the BW lysate. For the ACC_c lysate, NaOH removed up to 5 logs of LAL activity and for the ACC_t lysate, NaOH removed up to 4 logs of LAL activity. The BW lysate was not responsive to either the treated or untreated samples of LAL-RM or β-1,3-D-glucan. For the ACC_c lysate, NaOH reduced the LAL activity of LAL-RM and β-1,3-D-glucan by 1 log. The ACC_t lysate was non-responsive to either the untreated or treated samples of LAL-RM and LAL responsiveness was reduced by one log in the treated β-1,3-D-glucan samples. All three of the lysates-BW, ACC_c, ACC_t were responsive to equivalent preparations of untreated cotton dust (297, 6872, and 1162 EU/ml, respectively). NaOH removed all activity of the cotton dust preparations when evaluated with the BW lysate. With the ACC_c lysate up to 2 logs of LAL activity of the cotton dust preparations were removed and up to 3 logs of LAL activity were removed for ACC_t. These data would suggest that glucans are present in organic dust and the ACC lysates are sensitive to glucan. However, it appears that NaOH treatment does reduce the LAL activity of glucan and thus, at present NaOH treatment and use of a glucan sensitive lysate is not a method to qualitatively assess the contribution of glucan to organic dust.

INTRODUCTION

There are a variety of workplaces such as animal handling facilities, vegetable fiber processing plants, and waste processing plants, where workers are exposed to aerosols of organic dust. Exposure to these dusts have been associated with both acute and chronic respiratory disease. Endotoxin, a cell wall component of Gram negative bacteria, has been shown to correlate more closely with acute respiratory symptoms than gravimetric dust and recommendations have been made that endotoxin be measured in environments with organic dust.[1] However, organic dust is a heterogeneous mixture and other components of biogenic origin may contribute to the respiratory response as well as complicate the measurement of endotoxin. One such component is β-1,3-D-glucan a constituent of the cell wall of fungi. When inhaled both endotoxin and glucan activate a variety of cell systems resulting in a wide range of biological response.[2,3] Furthermore both components activate the system used to measure endotoxin, the Limulus amebocyte lysate (LAL) assay.[4] The LAL assay consists of a series of clotting enzymes (lysate) derived from the amebocyte (blood cells) of the horseshoe crab (Limulus sp.) which can be stimulated by endotoxin in a concentration dependent manner to form a clot. The lysate also contains a second enzyme system that can be activated by glucans. Therefore, if assaying an organic dust sample for endotoxin, it is possible that the results include a combined measure of endotoxin and glucan.

Differentiation between glucan and endotoxin can provide useful information in light of the cell stimulating properties associated with each component. A method that may provide a means to assess the contribution of glucan to LAL reactivity is by treatment of samples with NaOH. Sodium hydroxide has been shown to destroy endotoxin activity but not glucan activity.[5] In order to assess the contribution of glucan to an organic dust, test samples were treated with sodium hydroxide, neutralized with a buffer, and assayed by LAL from two different vendors, one which is known to react with both endotoxin and glucan and one that is non-responsive to glucan.

MATERIALS AND METHODS

All glassware was depyrogenated (PF) by dry heat for a minimum of 3 hours at >200° C. Disposable pipet tips were purchased sterile and assumed to be pyrogen free. Sterile water for injection was used for all extraction and dilution procedures. Individually packaged sterile microtiter plates (Corning) were used for the LAL analysis.

Endotoxin Analysis

A kinetic chromogenic LAL (lot #2L2180) from Biowhittaker (BW-Walkersville, Maryland) and two lysates from Associates of Cape Cod ACC-Woods Hole Massachusetts), a kinetic chromogenic LAL (ACC_c lot #01-12-103-C) and a kinetic turbidimetric LAL (ACC_t lot #99-97-440), were used to evaluate the samples. The LALs from ACC are reported to react with both endotoxin and LAL-RM while the LAL from BW is non-responsive to LAL-RM.

Evaluation of Comparability of Lysates

The BW LAL analysis kit includes a control standard endotoxin (CSE) which if reconstituted with PFW as specified, has a potency of 50 EU/ml for that specific batch of lysate. This potency is relative to the Reference Standard Endotoxin (RSE) of the U.S. Pharmacopeia, EC-5. Serial ten-fold dilutions over the range of 0.005-50 EU/ml of the BW-CSE (lot #2L2450) were prepared in PFW. ACC does not provide a separate CSE with their lysate; therefore, the ACC-CSE was purchased separately. Serial ten-fold dilutions over the range of 0.01-100 ng/ml of the ACC-CSE (lot #52) were prepared in PFW. One tenth of a milliliter of each dilution of both the BW and ACC-CSE was dispensed in the wells of the microtiter plate and assayed with the BW lysate (lot #2L2189), the ACC turbidimetric lysate (ACC_t lot #99-97-440), and the ACC chromogenic lysate (ACC_c lot #01-12-103-C) in a single assay.

As with the BW lysate, different lots of ACC lysate have different potencies for the same endotoxin. ACC have indicated that the potency of their lysate ranges between 5-10 EU/ng. For these analyses, a conversion factor of 10 EU/ng was used. This value was chosen because the conversion between nanograms and EU for the RSE is conventionally chosen as 10 EU/ng.[1] BW indicates a conversion factor of 10 EU/ng also applies to their lysate. However, it should be acknowledged that differences in the response of different lysates to a single sample could, in part, be related to one lysate having a potency of 5 EU/ng and another a potency of 10 EU/ng.

Evaluation of NaOH Concentration and Incubation Temperature on LAL Activity of LPS

A working solution of the LPS was prepared by suspending 10 milligrams of LPS in 100 milliliters of PFW. Two serial ten-fold dilutions were prepared and six 1 milliliter aliquots of the undiluted LPS and the 10^{-2} dilution were transferred to PF test tubes. Three of the tubes for each dilution were treated with 1 milliliter of 0.2 N NaOH (experimental samples) and the remaining tubes were treated with an equal volume of PFW and designated as control samples. The final NaOH concentration was 0.1 N. The 0.2 N NaOH was prepared each day by mixing 0.8 grams of NaOH in 100 milliliters of PFW. Each of the experimental and control samples were covered with Parafilm and vortexed one minute. One set of tubes from each experimental and control group were incubated for 24 hours at 4° C, room temperature, and 60° C respectively. After incubation, the samples were neutralized with 2 milliliters of Trizma® 2X buffer and analyzed with the LAL assay. The experiment was repeated using 0.4 N NaOH (1.6 grams of NaOH in 100 milliliters of PFW).

Evaluation of Trizma Buffer to Neutralize NaOH Treated Samples

The LAL endotoxin reaction is optimized in the range of pH 6.4 to 8.0 and LAL reactivity is suppressed if the pH of the sample and LAL deviates from this range. Therefore, an experiment was conducted to determine sample pH before and after the addition of the buffer.

The Trizma® buffer (tris(hydroxymethyl)aminomethane) of pH = 7.2 was made by mixing 1.8 grams of Trizma® HCL and 0.17 grams of Trizma® base (Sigma Chemical Company-St. Louis, Missouri) in 250 milliliters of PFW (Trizma® 1X buffer). The molarity of the solution was 0.05 M. One milliliter of the undiluted LPS solution was dispensed in 1 milliliter of 0.2 N NaOH or 1

milliliter of 0.4 N NaOH, neutralized with 2 milliliters of Trizma® 1X buffer, and the pH measured. The experiment was repeated using a Trizma® 2X buffer (93.6 grams of Trizma® HCL and 0.34 grams of Trizma® base in 250 milliliters of PFW. 0.1M). Trizma® 2X buffer was used for all experimental and control samples. The Trizma® 2X buffer was made up fresh each day and autoclaved after preparation. Control samples of the buffer were evaluated by LAL to determine if they were contaminated with endotoxin.

Evaluation of LPS, LAL-RM, β-1,3-D-Glucan, and Cotton Dust by the Different Lysates

Figure 1 shows the scheme used to evaluate the four samples with the different lysates. Four samples were evaluated for endotoxin and glucan: a commercially available lipopolysaccharide (E.coli 055:B5, lot #17F-40191-Sigma Chemical Co.), a sample of Limulus amebocyte lysate reactive material (LAL-RM; Biowhittaker, Walkersville, Maryland), a sample of β-1,3-D-glucan (Alpha-Beta Technology; Worcester, Massachusetts), and a sample of bulk cotton dust.

LPS

A working solution of LPS (E.coli 055:B5, lot #17F-40191) was prepared by dispensing 10 milligrams of LPS into 30 milliliters of PFW. Serial ten-fold dilutions through 10^{-3} were prepared by dispensing 0.5 milliliter aliquots of the LPS solution into 4.5 milliliter dilution blanks. Two 1 milliliter aliquots of the 10^{-3} dilution were transferred to PF test tubes, one aliquot designated as the experimental sample and the other as the control sample. A total of four samples were evaluated.

LAL-RM

A working solution of the LAL-RM was prepared by dispensing a 0.5 milliliter aliquot of the LAL-RM solution into a 4.5 milliliter dilution blank to yield a 10^{-1} dilution. Two 1 milliliter aliquots of the 10^{-1} dilution were transferred to PF test tubes, one aliquot designated as the experimental sample and the other as the control sample. A total of four samples were evaluated.

β-1,3-D-glucan

A particulate suspension of β-1,3-D-glucan was prepared by suspending 1 milligram of β-glucan in 1 milliliter of PFW. A ten-fold dilution was prepared by vortexing and then dispensing a 0.5 milliliter aliquot into a 4.5 milliliter dilution blank. Two 1 milliliter aliquots of the sample were transferred to PF test tubes, one aliquot designated as the experimental sample and the other as the control sample. A total of four samples were evaluated.

Cotton Dust

A 500 milligram sample of the cotton dust was suspended in 10 ml PFW in a 50 milliliter centrifuge tube and shaken for 60 minutes at room temperature. After extraction, the sample was centrifuged at 5000 rpm for 10 minutes. Serial ten-fold dilutions through a dilution of 10^{-3} of the supernatant were prepared. Two 1 milliliter aliquots of the 10^{-3} dilution were transferred to PF test tubes. One aliquot was designated as the experimental sample and the other as the control sample. A total of four samples were evaluated.

Sample Treatment

The two aliquots from each sample and replicate were treated as follows: One aliquot was treated with NaOH to inactivate the LAL activity of endotoxin and the second aliquot was treated with an equal volume of PFW. One milliliter of 0.2 N NaOH was added to each sample, which was covered with Parafilm, vortexed 1 minute, and incubated for 24 hours at 60° C. After incubation, the samples were neutralized with 2 milliliters of Trizma® 2X buffer.

The control samples were treated by the same procedure as the experimental samples except that an equivalent volume of PFW was substituted for the NaOH prior to incubation for 24 hours at 60° C. After incubation, the control samples were treated with 2 milliliters Trizma® 2X buffer.

Data Analysis

The samples were evaluated simultaneously using a microtiter plate reader. Software manufactured by Biowhittaker for the microtiter plate reader was used for endotoxin analysis with all three lysates. The slopes of the regression lines for the standard curves generated for each of the three lysates were evaluated for parallelism using a two-tailed T-test at a significance level of 0.05. A GLM-Anova was used to determine if there were differences between lysates for each type of sample and for the control and treated groups. Significant differences between means were evaluate by Duncan's test ($p=0.05$).

RESULTS AND DISCUSSION

Evaluation of Buffer Solution

Several preliminary experiments were performed to determine the appropriate NaOH and incubation temperature to inactivate the endotoxin as well as the appropriate strength of a buffer to neutralize the NaOH treated samples. The appropriate test sample pH is important because the LAL endotoxin reaction is optimized in the range of pH 6.4 to 8.0.[8] A sample pH above or below this optimal range may suppress the LAL reactivity.

The pH of LPS samples containing 0.2 N NaOH and 0.4 N NaOH was tested before and after neutralization with Trizma® 1X buffer. The pH for both NaOH concentrations after addition of the buffer ranged from 11.8-12.0. The experiment was repeated using a Trizma® 2X buffer to neutralize the samples. The pH was reduced to 10.2-10.6 for samples containing 0.2 N NaOH and 10.4-11.0 for samples containing 0.4 N NaOH. However these levels still exceeded the limits of the assay. To simulate the buffering capacity of the reconstituted lysates, an equal volume of Trizma® 1X buffer was added to the samples buffered with Trizma® 2X buffer. The pH of the samples was lowered to approximately 8.0 which is at the upper limit of acceptability for the assay. Both the BW and ACC lysates contain a buffer. BW did not specify the buffer concentration of their lysate however, ACC indicated that the concentration of their buffer was 0.2 M. The molarity of the ACC buffer was greater than the molarity of the Trizma® buffer used to simulate the lysate buffering capacity (0.05 M), therefore, the final pH of the NaOH treated solutions determined by the simulation is conservative. For all subsequent analyses, the samples were buffered with Trizma® 2X buffer and the buffering capacity of the lysate was relied on to reduce the pH to an acceptable range. The pH of the actual samples assayed could not be tested due to the risk of contamination and because there was not enough volume to take a representative amount which to test the pH.

Evaluation of NaOH Concentration and Incubation Temperature

A second preliminary experiment was conducted to determine the appropriate NaOH concentration and incubation temperature to inactivate the LAL activity of LPS. A previous study had demonstrated that 0.2 N NaOH at room temperature inactivated the LAL activity of LPS.[1] However, room temperature may not be satisfactory for organic dust samples. While treatment at room temperature is satisfactory for NaOH treated LPS samples it would not be satisfactory for control (non-NaOH treated) samples of organic dust due to the chance of microbial growth in the samples. Therefore, in addition to room temperature, treatments at 4° C and 60° C were evaluated as alternatives to control microbial growth and have an effective treatment. The NaOH concentrations evaluated were 0.2 N and 0.4 N.

All evaluations were done with the BW lysate. The results of these experiments are presented in Figure 2. Neither the 4° C nor the room temperature treatments effectively removed the LAL reactivity. Only for the treatment at 4° C was a difference observed from the effect of NaOH concentrations. This differs from published data which indicate that treatment with 0.2 N NaOH at room temperature for 24 hours removes the LAL activity of LPS[1].

The treatment at 60° C removed all LAL activity at both levels of NaOH. Therefore, for subsequent experiments 0.2 N NaOH at 60° C for 24 hours was used to remove LPS. An additional benefit in using 0.2 N NaOH is that it takes less buffer to neutralize the test samples to a range of pH 6.4-8.0.

Evaluation of Comparability of Lysates

In order to compare results of identical samples assayed with different lysates, equivalence between the lysates must be demonstrated. The plots of the regression lines for the BW-CSE assayed with each of the three lysates are shown in Figure 3. The slopes of the curves for all three lysates were significantly different (Table 1, $p<0.05$), suggesting that comparison of responses for different lysates is not valid. However, the mean square errors for each line ($BW=0.0000042$, $ACC_c=0.000012$, $ACC_t=0.0000041$) and the confidence intervals were small suggesting that there may be no practical difference in the slopes for the three lysates. Therefore, while there may be a difference in the response of a sample to the three lysates that is due to variability between lysates, it appears from the analysis of the BW-CSE that these differences are small and comparisons of the response of different samples to the three lysates can be useful.

Analysis Using Different Lysates

Summary data describing the evaluation of LPS, LAL-RM, β-1,3-D-Glucan, and cotton dust by the different lysates is given in Table 2.

LPS

The responses of the three lysates to a single dilution of a purified LPS are shown in Figure 4. The Anova demonstrated that there was a significant difference in the response of the lysates and visual inspection of the data suggested that there was a difference between each of the three lysates. The post-hoc test of the means demonstrated significant differences between the BW and ACC_c and the BW and ACC_t lysates. There was no difference between the ACC_c and the ACC_t lysates. The CVs for the analysis of replicate samples of LPS for each lysate ranged from 30-47%. The high CVs are of concern and may reflect a combination of experimental error and surface phenomenon within each vessel. Purified LPS is hydrophobic and when suspended in PFW may preferentially adsorb to the surface of the sample container or form micelles that respond differently to the lysate.[6] Similar surface phenomenon may not occur with a CSE, due to treatment of the preparation by the vendor, or for samples of organic dust which contain many substances that would compete with the LPS for adsorption sites.
There was no measurable LAL response for the LPS samples treated with NaOH using the BW lysate. While a response was observed for both the ACC_t and ACC_c lysates, the treatment effectively removed greater than 4 logs LAL activity.

LAL-RM and β-1,3-D-glucan

Figures 5 and 6 show the results of the LAL-RM and β-1,3-D-glucan samples assayed with the three lysates. One form of LAL-RM is a β-1,4-D-glucan derived from hollow-fiber hemodialyzers with a saponified cellulose acetate membrane, whereas β-1,3-D-glucan is derived from the cell wall of fungi, yeast, and algae.[5] The BW lysate was not responsive to either the LAL-RM or β-1,3-D-glucan. These data are in agreement with the information provided by BW which specifies that their lysate is specifically treated to remove responsiveness to glucan.[5]

The ACC_c was responsive to LAL-RM. This is in agreement with information provided by the vendor. However, the ACC_t, also reported to be LAL-RM sensitive[5], had little responsiveness to LAL-RM. Both ACC lysates were responsive to β-1,3-D-glucan, however the response of the ACC_c lysate was significantly greater than the response of the ACC_t lysate, suggesting a greater reactivity to substances which activate the glucan enzymatic pathway in the ACC_c lysate.

NaOH removed up to one log of the LAL activity of the LAL-RM for the ACC_c lysate. For β-1,3-D-glucan, NaOH treatment removed one or more logs of LAL activity for both the ACC_c and ACC_t lysates. NaOH treatment has not been shown to decrease the LAL activity of β-1,3-D-glucan, however, it has been shown to alter its molecular conformation (triple helix to single chains) which resulted in an increase in LAL reactivity.[7] The molecular configuration of the β-1,3-D-glucan is source dependent. The confifiguration of the β-1,3-D-glucan used in this study is not known and it is possible that treatment with NaOH altered the configuration which resulted in a decrease in LAL activity, however, further studies are needed to confirm this hypothesis.
Cotton Dust.

The response of cotton dust samples assayed by the three lysates is shown in Figure 7. There was a significant difference in the response of identical samples of untreated cotton dust extracts for the three lysates. Organic dust likely contains both glucan and endotoxin, therefore the differences seen with the ACC_t and BW lysates could be accounted for by the presence of glucan. Cotton dust also contains large amounts of cellulose (β-1,4-D-glucan) which potentially may be a source of LAL-RM. This could account for the difference in the ACC_c lysate (responsive to endotoxin, LAL-RM and the β-1,3-D-glucan) and the ACC_t lysate (responsive to endotoxin and β-1,3-D-glucan).

The BW lysate was not responsive to the treated cotton dust samples. For the ACC lysates treatment with NaOH reduced, but did not eliminate the LAL activity. This finding is consistent with the presence of either β-1,3-D-glucan or LAL-RM in the cotton dust. However, because the LAL activity of the β-1,3-D-glucan or LAL-RM used in these studies is partially inactivated by the NaOH treatment, it is not possible to quantitatively assess the contribution of glucan to organic dust by this technique and we can only speculate about the qualitative contribution.

CONCLUSIONS

This study found a small but significant difference in the response of the three lysates to a single control standard endotoxin (CSE). This finding, if confirmed by additional studies, suggests that comparison of endotoxin results between laboratories is valid only if LAL from the same vendor is used.

There was a large variability between samples of purified LPS evaluated simultaneously by the three lysates. These differences may be related to the hydrophobic state of the LPS, however, other unidentified differences between the lysates cannot be excluded.

The data suggest that LAL-RM and β-1,3-D-glucan may contribute to the LAL responsiveness of cotton dust, however the concentrations of these materials could not be determined by the techniques used in this study.

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Table 1. Regression Data of Standard Curves

CSE/lysate	Slope	Intercept
BW CSE/BW	-0.173 A	3.103 A
BW CSE/ACC_t	-0.186 B	3.06 B
BW CSE/ACC_c	-0.197 C	2.94 C

BW = Biowhittaker
ACC_t = Associates of Cape Cod turbidimetric lysate
ACC_c = Associates of Cape Cod chromogenic lysate
CSE = Control Standard Endotoxin
Note: Values followed by the same letter are not significantly different, p = 0.05.

Table 2. Summary Results of LAL Analysis

Sample	CONTROL Mean EU/ml	CV	Dun-can's*	EXPERIMENTAL Mean EU/ml	CV	Dun-can's*	Reduc-tion %
Lps[A]	64626	0.36	A	0	0.00	A	100
Lps[B]	249818	0.47	B	53	0.65	B	99.99
Lps[C]	162389	0.30	B	49	0.93	B	99.97
LAL-RM[A]	0	0.00	A	0	0.00	A	0
LAL-RM[B]	2138	0.12	B	13	0.39	B	99.39
LAL-RM[C]	0.5	0.06	C	0.06	0.67	C	89.29
Glucan[A]	0	0.00	A	0	0.00	A	0
Glucan[B]	5191	0.07	B	531	0.41	B	89.77
Glucan[C]	1151	0.31	C	22	0.84	C	98.08
CD[A]	4762	0.14	A	0	0.00	A	100
CD[B]	109944	0.58	B	1241	0.21	B	98.87
CD[C]	18598	0.10	C	91	0.59	C	99.51

Note: Four replicates were evaluated for each sample-lysate combination
[A]BW lysate
[B]ACC chromogenic lysate
[C]ACC turbidimetric lysate
*Means followed by the same letter are not significantly different; $p < 0.05$.

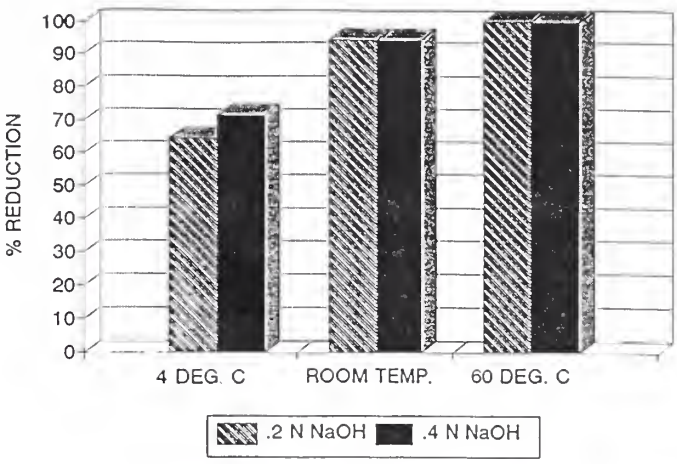

Figure 2. Effects of Temperature and NaOH on LPS

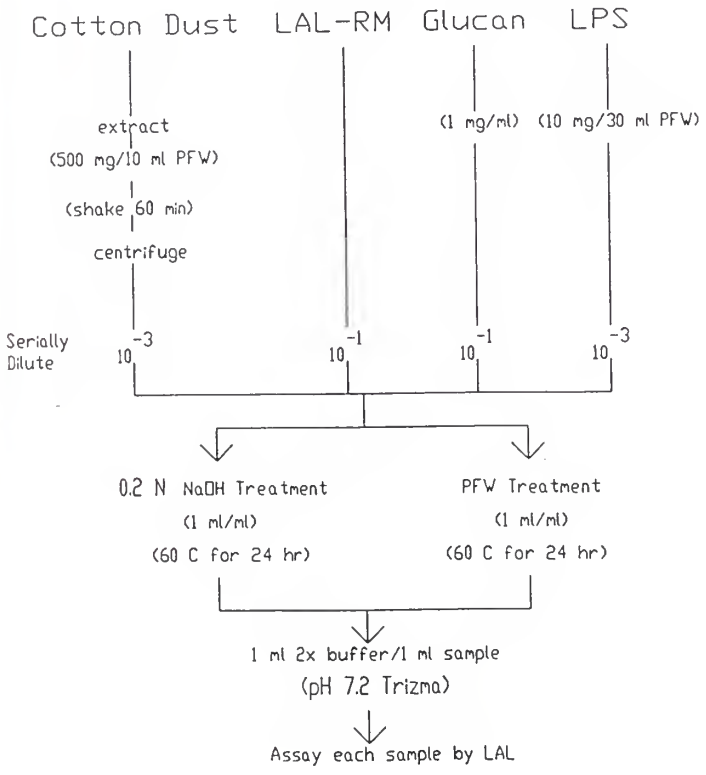

Figure 1. Sample Preparation Scheme

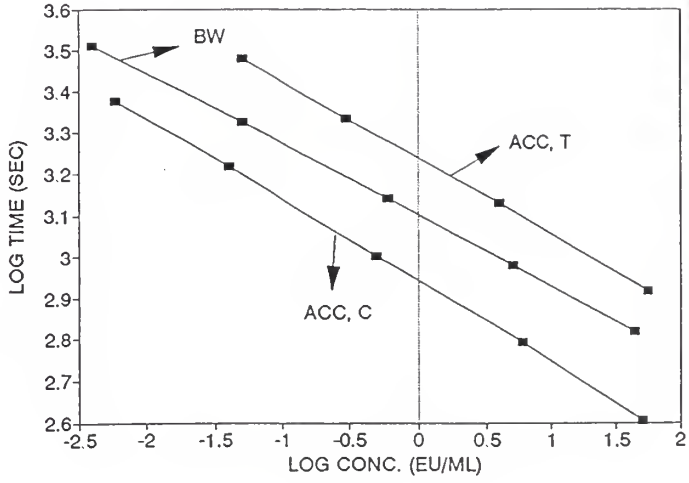

Figure 3. Comparison of Standard Curves

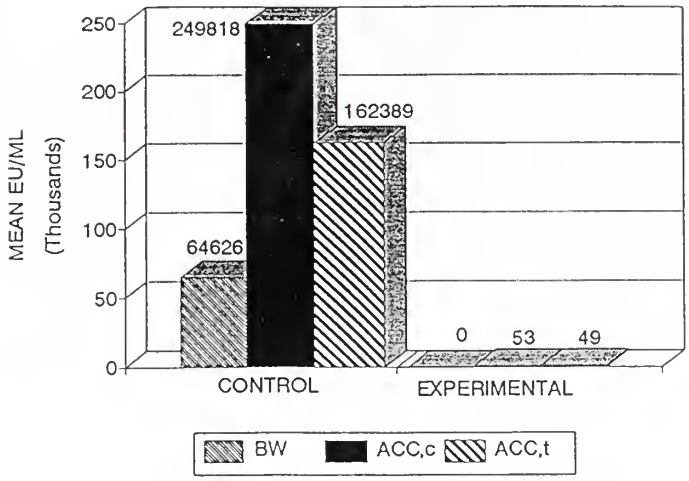

Figure 4. LPS Samples Assayed with 3 Different Lysates (Experimental = NaOH Treated)

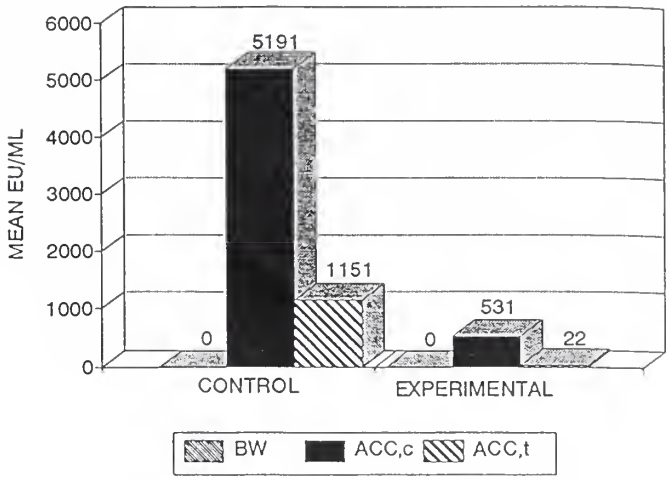

Figure 6. Beta-1, 3-D-Glucan Samples Assayed with 3 Different Lysates (Experimental = NaOH Treated)

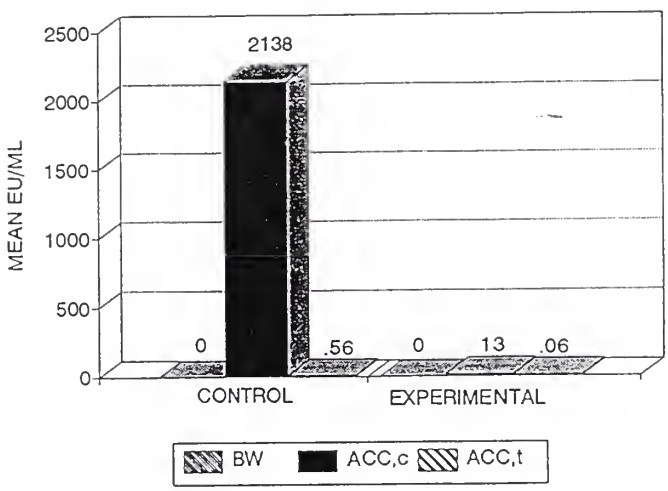

Figure 5. LAL-RM Samples Assayed with 3 Different Lysates (Experimental = NaOH Treated

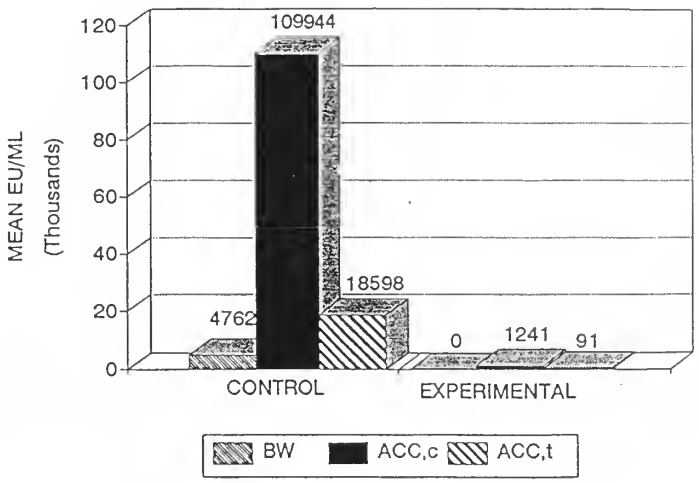

Figure 7. Cotton Dust Samples Assayed with 3 Different Lysates (Experimental = NaOH Treated)